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Traumatic brain injury (TBI) reduces cerebral blood flow (CBF) and renders the brain vulnerable to secondary ischemia. Hypotension contributes to poor outcome after TBI in humans. We have prevented hypoperfusion and restored autoregulation after TBI. The goals of this project are to determine whether treatment based on our observations will prevent CBF reductions, brain edema and histological damage after TBI and hemorrhagic hypotension and to understand the mechanisms that contribute to the efficacy of the proposed treatments.

Specific Aim 1 addressed the hypothesis that impairment of cerebrovascular function will result in brain injury after TBI and hemorrhagic hypotension that would not occur after hypotension alone.

Specific Aim 2 addressed the hypothesis that post-TBI cerebral hypoperfusion is caused by nitric oxide (NO)-dependent mechanisms.

Specific Aim 3 addressed the hypothesis that increased production of superoxide during TBI and subsequent hypotension/resuscitation is responsible for the impaired cerebrovascular reactivity.

Specific Aim 4 will address the hypothesis that small volume resuscitation with hypertonic saline will restore cerebral circulatory and systemic hemodynamics without causing the pronounced changes in brain water diffusion seen after TBI and hypotension/resuscitation with shed blood.

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INTRODUCTION:

Abbreviations are defined in the text with first usage but are also defined below:

AUC	area under the curve
CBF	cerebral blood flow
CBV	cerebral blood volume
CB1	cannabanoid receptor type 1
CGRP	calcitonin gene-related peptide
FID	free induction decay
FPI	fluid percussion traumatic brain injury
LDF	laser Doppler flowmetry
MAP	mean arterial blood pressure
MCA	middle cerebral artery
MRI	magnetic resonance imaging
NO	nitric oxide
NOS	nitric oxide synthase
TBI	traumatic brain injury
TT	transit time

Traumatic brain injury (TBI) reduces cerebral blood flow (CBF) and renders the brain vulnerable to secondary ischemia. There is clinical evidence that hypotension contributes to poor outcome after TBI, likely because of traumatic damage to the cerebral circulation. Our research has resulted in novel and important observations related to the mechanisms of traumatic vascular injury. In the first year of the funding period we observed that posttraumatic hypoperfusion can be prevented using substrates of nitric oxide synthase (NOS). We also observed that NOS enzyme activity is not affected by our model of TBI, suggesting that TBI is affecting NO directly. We also reported that TBI decreases CBF in rats treated with inhibitors of NOS suggesting TBI reduces CBF by mechanisms in addition to the destruction of NO. We observed that prolonged (over 45 minutes) superoxide anion radical production occurs after TBI and that posttraumatic hypoperfusion can be significantly reduced using oxygen radical scavengers. In addition, we reported that while agonist-induced vasodilation and vasoconstriction is intact in rat cerebral arteries after TBI, myogenic responses to hypotension are significantly reduced by trauma. In the past year we have made additional discoveries that will contribute to further understanding of the pathophysiology of TBI. We have made progress with the magnetic resonance imaging (MRI) measurements of CBF and we have calculated quantitative, sequential, regional CBF values that are comparable to those reported using other techniques. We have modified of techniques for producing progressive, controlled hemorrhage to allow us to perform survival studies in rats after TBI and hemorrhage. These studies will allow us the assess the behavioral effects of TBI as well as the effects of experimental TBI on CBF and histopathology. We have observed that L-arginine does not restore myogenic responses after TBI, a result that is consistent with earlier studies demonstrating that CBF autoregulation is not an NO-dependent response. We have noted that TBI does not reduce the density of calcitonin gene-related peptide (CGRP)-containing perivascular nerve fibers. CGRP, one of the vasodilatory transmitters that are containing in the nerve plexus that surrounds the cerebral circulation, has been suggested to mediate autoregulation [1]. Our observations suggest that TBI does not impair autoregulation by reducing the density of these vasodilatory CGRP-containing nerve fibers. In contrast to these negative results, we have exciting new data suggesting that perivascular nerve fibers may modulate myogenic responses through the vasodilatory cannabanoid receptors. We observed that the cannabanoid 1 (CB1) receptor antagonist SR141716A significantly reduces myogenic responses to decreased transmural pressure in isolated middle cerebral arterial (MCA) segments. We have noted that phenol, which destroys perivascular nerve fibers, also reduces myogenic responses suggested that perivascular nerves may release an agent such as anandamide which dilates the cerebral circulation by acting at CB1 receptors. We have completed a dose-response study using an arginine-containing hypertonic

solution to improve CBF during resuscitation from hemorrhagic hypotension after TBI. A hypertonic saline solution containing 100 mg/kg of L-arginine most effectively restored CBF after hemorrhage. We are continuing these studies to compare hypertonic arginine solutions to other resuscitation solutions such as lactated Ringer's or hypertonic saline.

These exciting observations, which are described in detail below, are consistent with the overall goal of this project: to determine whether a treatment strategy based on our observations will prevent CBF reductions, brain edema and histological damage after TBI and hemorrhagic hypotension as well as to understand the mechanisms that contribute to the efficacy of the proposed treatments.

BODY:

This section will summarize progress made in the previous year as related to each **Specific Aim**.

Specific Aim 1 is to address the hypothesis that impairment of cerebrovascular function will result in brain injury after TBI and hemorrhagic hypotension that would not occur after hypotension alone. We will relate changes in CBF with MRI evidence of brain edema in rats with and without TBI and hypotension/resuscitation.

SA 1.1 - Quantitative CBF measurements using MRI.

Quantitation of CBF using intravascular tracers requires knowledge of the input function as the shape of the intracerebral transit curves are affected by the history of the bolus prior to arriving to the brain [2]. We have shown that common pharmacological and physiological manipulations alter the input function [3] yielding erroneous interpretations of relative CBF unless these input function changes are considered. In that paper, we validated the use of both the venous and arterial residual transit curves as components of the input function-the area under the curve (AUC) of a representative pixel in a cerebral vein was utilized as the denominator in the estimation of the cerebral vascular fraction [3]. Transit time estimation of the input function came from a cerebral artery [3]. We showed that this method correctly detected changes in CBF after pharmacologic manipulations, but grossly overestimated the actual cerebral vascular fraction and produced inaccurate absolute CBF values. More recent efforts have incorporated the input function differently [4] to yield quantitative CBF values. We report here a new way to incorporate the input function that obviates the need for an arbitrary correction factor, yields reasonable estimates of vascular fractions and results in CBF values within the range reported by other methods. Specifically, we have included the sum of all pixels identified as a cerebral vein as the venous residue function. Summing these pixels seems to satisfactorily represent the bolus of contrast agent presented to the brain and a more physiologically accurate denominator for the determination of brain vascular fraction.

For these experiments, rats (n=13) were anesthetized with isoflurane, intubated, injected intravenously with gadolinium DTPA, and bolus transit curves obtained with a fast gradient echo as reported [3]. Body temperature was maintained with a warm water blanket. For the input function, veins and arteries visible on the same slice used for the brain transit curves were located. These regions were hyperintense at baseline and decreased and recovered intensity in temporal sequence corresponding to the artery first, the brain second and the vein last. The artery was used for transit time (TT) calculations. The bright region in the baseline image that subsequently darkened with bolus passage representing a vein was outlined by hand, and the area under this entire venous curve (AUC) used later in the calculation of vascular volume fraction. Mixed gray/white brain regions in both hemispheres were analyzed for the results reported here. Operator-defined arithmetic integration of the transit curves was used instead of fitting the curve to a gamma variate function because many of the residue curves, particularly those from the veins, appeared to be composed of multiple compartments and recirculation. These curves then were analyzed for mean transit time (from the arterial and brain parenchymal curves) and the brain

vascular fraction (from the AUC of the venous and parenchymal curves). True TT was obtained by subtracting the TT obtained in the artery from that obtained in the brain, and the vascular volume fraction was obtained by the ratio of the mean AUC in the brain to the AUC under the transit curve of the sum of the venous pixels. The vascular fraction was converted to ml/g, using a previously determined intensity/concentration function [3] and the density of brain vs. blood. CBF was then calculated as the vascular fraction/True TT (multiplied by 60 sec/min), and then expressed per 100g brain tissue. The resulting units were ml/min/100g.

Because of limited spatial resolution, the arterial curve was sometimes difficult to reliably locate and often included a small tail that likely represented volume-averaging with adjacent brain or vein, but the curve could be identified in most cases. Susceptibility artifacts also blurred the borders of the cerebral veins and the identification method was subsequently modified to include only those pixels as venous that clearly darkened with bolus passage. Despite these difficulties, the model yielded reasonable results (see table):

Baseline CBF (ml/min/100g)	Baseline TT (sec)	Baseline CBV (ml/100g)
117 ± 14	0.89 ± 0.10	1.49 ± 0.14

The original tracer kinetic models were applied to other methods, such as radioisotope methods, in which external detection or artificial reference organs readily yield values related to the total amount of contrast agent presented to the brain. Alternatively, in our previous approach, using a representative voxel for the input function, while valid for relative measurements, was not sufficient for quantifying the vascular fraction. By summing the venous voxels, the apparent vascular volume fraction using this method is closer to actual vascular fraction than using single pixel values and CBF is within values obtained using radioactive microspheres in rats anesthetized with isoflurane [5]. We are currently using this model to measure CBF, CBV and TT during and after TBI and hypotension.

SA 1.2 - Quantitative CBF measurements after TBI and hypotension in rats: MRI studies

Male Sprague-Dawley rats were surgically prepared for MRI CBF measurements and TBI, as described below, and randomly split into four groups to determine the individual and combined effects of mild hypovolemic hypotension and TBI on cerebral blood flow using the technique of contrast agent bolus-tracking MRI.

Male Sprague-Dawley rats (452±10g, n=26) were anesthetized with 4% Isoflurane in O_2:air (50:50), intubated and mechanically ventilated at an Isoflurane concentration of 2% in O_2:air (20:80) at a rate of 20-30 bpm. Two femoral arteries and one femoral vein were cannulated with PE90 tubing containing heparinized plasmalyte (5 U/ml). One arterial line was used from then on for continuous monitoring of MAP. Core temperature was maintained using a circulating water bath/blanket and monitored continuously via a rectal temperature probe. After placement in a stereotactic holder, a midline incision was made in the scalp. The scalp and underlying fascia were retracted for a 5 mm outside diameter craniotomy (midway between Lambda and Bregma, 3.5 mm to the right of the midline suture). Once the craniotomy had been cleared, a machined trauma adapter made from a 20 gauge needle hub was cemented over the craniotomy using superglue and dental acrylic. The adapter was filled with saline and attached to the fluid transmission tube, which allows for continuous transmission of the injury pulse from the fluid percussion injury device to the right cortex of the rat. The animal was wrapped in a water blanket for the remainder of the experiment. At this point a blood sample was taken to ensure that hemodynamic parameters were within acceptable limits. MAP was maintained between 95 and 105 mmHg by adjustment of the isoflurane concentration, typically between 1.4 and 1.0%.

After preparation, the rats were placed in a specially designed plexiglas cradles and the cradle was placed in the magnet (Oxford Instruments, 4.7T), the coil tuned to the proton frequency (200.056 MHz) and the magnetic field throughout the sample volume was then maximized by shimming on the water free induction decay (FID). Once a series of pilot images had been acquired to allow for planning of the transverse imaging slices, a high resolution image

was acquired and the FPI device was calibrated and connected to the trauma adapter. Another high resolution image was then acquired, followed by a DWI experiment (three diffusion weighting of 0, 3 and 5 gauss/cm, tr = 3 sec, nv = 128, nt = 1) and a high resolution reference movie (TE = 0.003 sec, tr = 0.008 sec, nv = 128, nt = 2). Randomization of the experimental groups (see below) then took place by blind selection of a labelled slip of paper.
Group One = TBI + 45 minutes hypovolemic hypotension at 60 mm Hg
Group Two = TBI
Group Three = Sham injury + 45 minutes hypovolemic hypotension at 60 mm Hg
Group Four = Control (sham injury)

The bolus-tracking movie was then conducted using the same set of acquisition parameters except that nv and nt were reduced to 64 and 1 respectively, producing a total acquisition time for each frame of 0.512 sec. A bolus of 0.3 ml contrast agent (OMNISCAN, gadodiamide) was introduced during the sixth frame of this movie and took approximately 1.5 seconds for the total injection through the femoral vein. After each bolus-tracking movie a washout period of 20 minutes was used to reduce build-up of the contrast agent via renal filtration and excretion. After the washout period, the MAP was lowered to 60 mm Hg by removal of whole blood from one of the femoral arterial lines. Another reference movie followed by a bolus-tracking movie was then conducted, immediately after which the blood was re-infused to re-establish MAP at 100 mmHg. A washout period followed, then a DWI and another reference + bolus-tracking movie pair. Fluid percussion induced TBI took place (Groups One and Two) after the washout, followed immediately but another reference + bolus-tracking movie pair. At the end of the washout period, MAP was once again lowered to 60 mm Hg by removal of whole blood and another reference + bolus-tracking movie pair acquired. MAP was maintained at 60 mm Hg for Groups One and Three, while for rats in Groups Two and Four, it was restored to 100 mm Hg as above. A DWI was then conducted at the end of the washout and then another reference + bolus-tracking movie pair acquired. For Groups One and Three, the MABP was then restored to 100 mm Hg as above. A final DWI and reference + bolus-tracking movie pair were then acquired to determine the results of resuscitation.

K-space filling of the bolus-tracking movie using the reference movie was performed to allow for enhanced spatial resolution. Movies were then constructed in a file format for use in an on-site developed software package (Transit) and analyzed using the following methodology. An artery and vein at the base of the ipsilateral hemisphere of the brain were selected to provide the input and post-cerebral vascular functions respectively. From this data the passage time (To-TOA) of the bolus in seconds, as well as the total signal observed were determined for each vessel. The majority of the parenchyma of the brain was then outlined and the average flow curve determined to give the AUC and To-TOA.
This data was then used to calculate the: TT (in seconds), CBV (expressed as percent of total brain volume), and CBF (expressed as ml/100 g tissue/min).

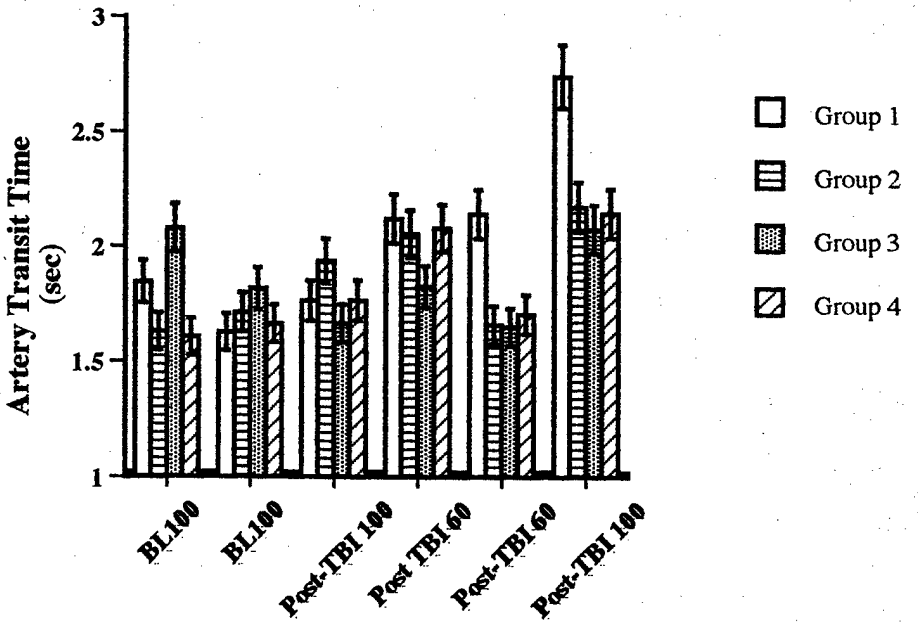

Figure 1 - Arterial transit time in rats after TBI and hemorrhagic hypotension. (Grp 1), TBI only (Grp 2), hypotension only (Grp 3) or sham operated (Grp 4).

TT for the bolus increased in all groups during the control and post-TBI hypotensive periods (mv2 and mv2b respectively). This could indicate a lower input function or that a greater proportion of the total blood volume leaving the heart is being shunted to the cerebral circulation or that the arteries are in vasoconstriction.] All four groups exhibited the same behavior until mv2c, where it can be seen that Group One's arterial transit times remained higher than the other three groups (which exhibited the same end value) for the remainder of the experiment.

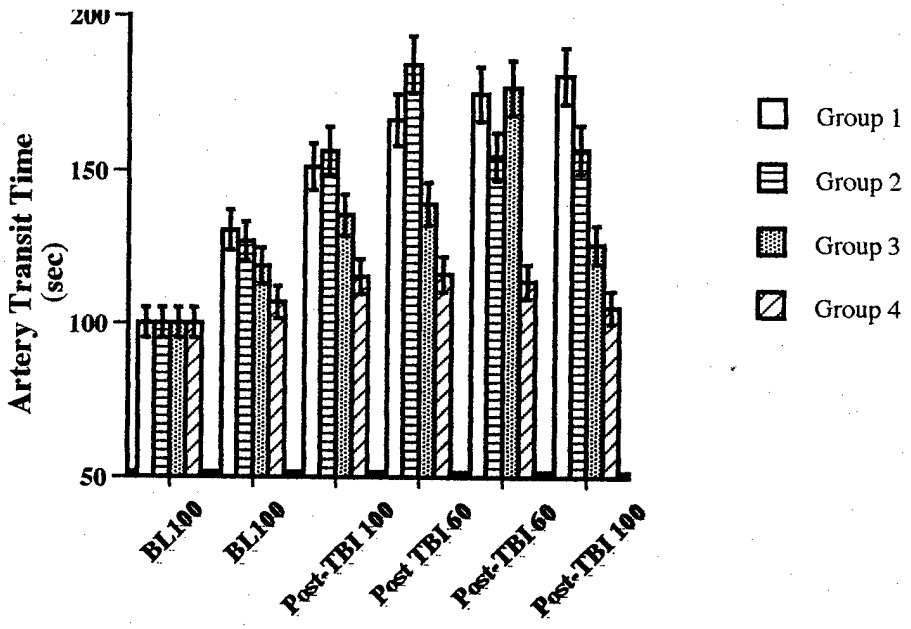

Figure 2 - Venous total TT in rats after TBI and hemorrhagic hypotension. (Grp 1), TBI only (Grp 2), hypotension only (Grp 3) or sham operated (Grp 4).

As Figure 2 illustrates, there was a general trend, for Groups One, Two and Three to increase over the experiment, whereas Group Four showed no change over the same time period. The differences observed between the groups were greatest at mv2c, where Group One showed a significant difference ($p < 0.02$) to the control group. Group Two showed a change in venous TT relative to Group Four that approached statistical significance ($p < 0.06$). The were no significant differences between Groups Three and Four.

The measured AUC for the venous blood flow curves (data not shown) showed no differences or no discernible pattern of behavior for any individual or between the groups throughout the entire experiment.

Whole brain TT behaved the same for each of the four groups, showing an overall increase with time, showing no significant differences between any of the groups. One point to note is that the passage time of the bolus through the cerebral parenchyma increases with each of the hypotensive episodes. The two groups that experienced the 45 minutes of hypotension showed a continued increase in whole brain TT, while the control and TBI only groups showed a reduction in TT after returning to baseline systemic blood pressures.

Whole brain TT increased in the control group (Grp 4) during the initial movie during hypotension but the decreased during continued hypotension and reinfusion. In contrast, in the TBI + hypotension group (Grp 1) TT increased steadily after TBI and hypotension and reinfusion. The groups subjected to TBI only (Grp 2) or hypotension only (Grp 3) exhibited whole brain TT's between the control and TBI + hypotension group (Figure 3, next page).

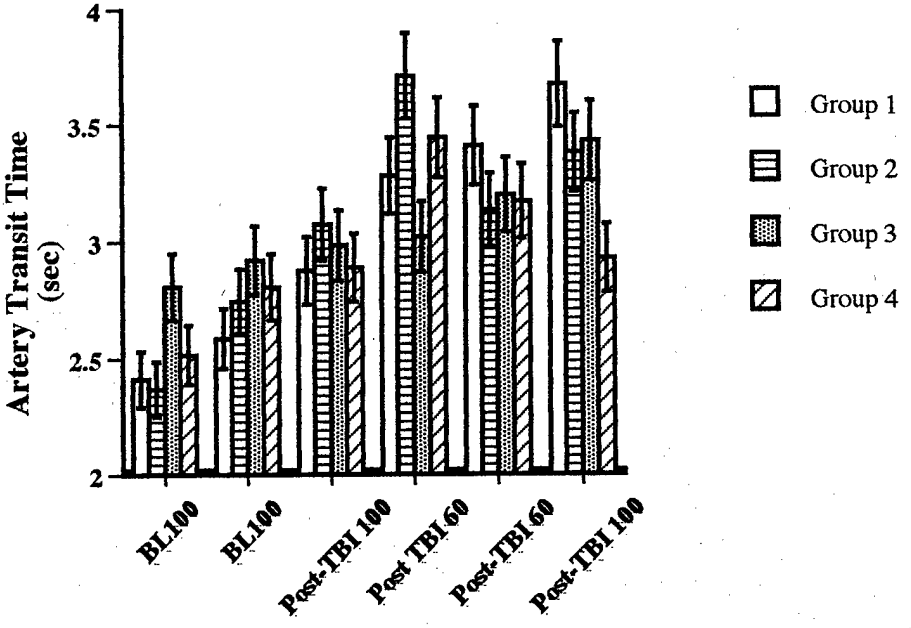

Figure 3 - Whole brain total TT in rats after TBI and hemorrhagic hypotension. (Grp 1), TBI only (Grp 2), hypotension only (Grp 3) or sham operated (Grp 4).

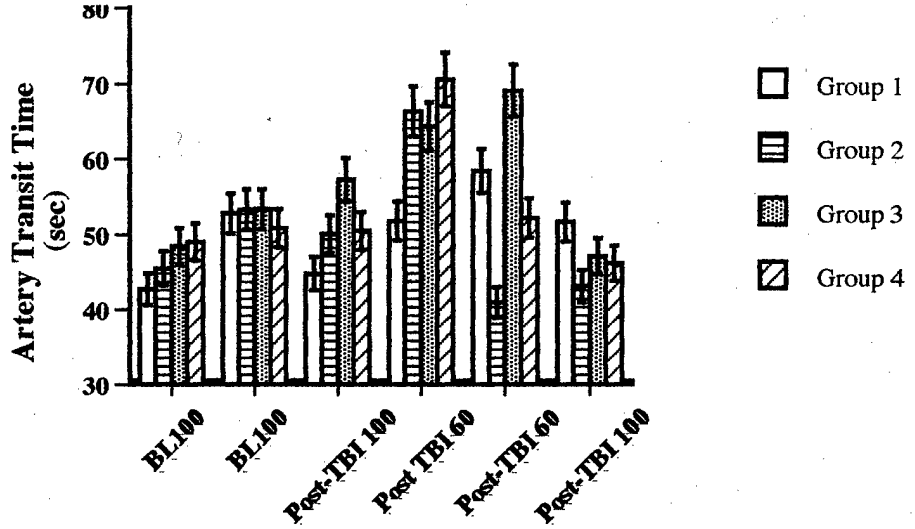

Figure 4 - whole brain AUC in rats after TBI + hemorrhagic hypotension. (Grp 1), TBI only (Grp 2), hypotension only (Grp 3) or sham operated (Grp 4).

The area under the tracer transit curve increased markedly in the sham operated group (Grp 4) immediately after the onset of hypotension but then returned to baseline during continued resuscitation and reinfusion. In contrast, whole brain AUC in TBI+hemorrhage group (Grp 1) increased gradually during hypotension and then returned towards baseline during resuscitation. These observations would be consistent with a rapid increase in tracer volume in the sham group (i.e. autoregulatory vasodilation and increased blood volume) but a much slower response to hypotension in the TBI+hypotension group. Together, these studies indicate that arterial and venous responses to hemorrhage are altered but not abolished by moderate TBI. We are currently investigating the effects of higher levels of TBI on TT, AUC, CBV and CBF.

Specific Aim 2 is to address the hypothesis that post-TBI cerebral hypoperfusion is caused by

studies were described in detail in our 1997 progress report and the manuscript has been included as an appendix to this report. More general goals of this section of the application include an investigation of the role of NO and other potential cerebral vasodilatory neurotransmitters in normal cerebral vasodilatory responses as well as in the impaired cerebral vasodilation that occurs after TBI [89]. The first set of studies (*SA 2.1 - Myogenic responses after TBI: effects of treatment with L-arginine*) presented address the hypothesis that TBI-induced reductions in NO might contribute to reduced vasodilatory responses to hypotension in isolated cerebral arteries after TBI and that the addition of L-arginine may restore these responses.

The second set of studies (SA *2.2 - Microdialysis measurement of cerebral nitric oxide levels)* describes initial experiments demonstrated that cerebral NO levels can be monitored using microdialysis in conjunction with an Antek NO Detector which was purchased with funds from the Department of Anesthesiology at UTMB.

The third set of investigations (SA *2.3 - Effects of peroxynitrite on myogenic responses in isolated, pressurized middle cerebral arteries)* address the hypothesis that NO is not simply inactivated after TBI but is converted to the active oxidant, peroxynitrite, which is responsible for impaired vasodilatory responses.

The fourth set of experiments (*SA 2.4 - Effects of TBI on CGRP-containing perivascular nerve density.*) was to test the hypothesis that a vasodilatory neurotransmitter other than NO might be involved in impaired vasodilation to hypotension after TBI. CGRP, vasoactive transmitter contained in nerves that surround the cerebral circulation, has been implicated in autoregulatory responses to hypotension [1]. The density of CGRP-containing perivascular nerves was counted after moderate and severe TBI to determine whether TBI affected the number of these potentially regulatory nerve fibers.

The fifth set of experiments (*SA 2.5 Myogenic responses to hypotension: Possible role of anandamide and perivascular nerves*) was designed to investigate another vasodilatory agent that might be important for the control of the cerebral vasculature normally as well as after traumatic or ischemic brain injury. These experiments demonstrated that the cannabanoid 1 receptors may be important in autoregulatory vasodilation and, therefore, may be damaged by TBI and/or subsequent hypotension.

SA 2.1 - Myogenic responses after TBI: effects of treatment with L-arginine

We have reported that L-arginine (100mg/kg) completely prevents posttraumatic hypoperfusion in rats [10]. In addition, we have reported that TBI reduces myogenic responses in isolated, pressurized MCA segments in rats harvested 5 or 30 minutes after moderate, central fluid percussion TBI [11]. In order to determine whether L-arginine would improve myogenic responses, Male Sprague-Dawley rats weighing 350-400 g were anesthetized with isoflurane in an anesthetic chamber, intubated, and mechanically ventilated with 1.5-2.0% isoflurane in O_2:room air (70:30) using a volume ventilator (EDCO Scientific, Chapel Hill, NC). Polyethylene cannulae were placed in a femoral artery and vein for drug infusion and arterial pressure monitoring, respectively. Rectal temperature was monitored using a telethermometer (Yellow Springs Instruments, Yellow Springs, OH) and maintained using a thermostatically controlled water blanket (Gaymar, Orchard Park, NY). Rats were prepared for midline fluid-percussion TBI as previously described [12]. Briefly, the rats were placed in a stereotaxic frame and the scalp was sagittally incised. A 4-mm hole was trephined into the skull over the sagittal suture approximately midway between lambda and bregma and a modified LuerLok syringe hub was placed over the exposed dura and bonded in place with cyanoacrylic adhesive and covered with dental acrylic. Isoflurane was lowered to 1.5%; the rats were connected to the trauma device, and randomly assigned to one of the groups listed below.

After TBI or sham injury, anesthetized rats were decapitated, the brains were removed and the middle cerebral arteries were harvested. Cerebral arteries were mounted in an arteriograph as described [13]. Briefly, a section (2 mm) of the artery was mounted in the arteriograph by inserting micropipettes into the lumen at either end and securing the vessel with nylon suture (10 0). The mounted arterial segments were bathed in physiological salt solution (PSS) of the following composition (mM : NaCl, 130; KCl, 4.7; $MgSO_4 \cdot 7H_2O$, 1.17; glucose, 5; $CaCl_2$, 1.50, $NaHCO_3$,

15. When gassed with a mixture of 95% air and 5% CO_2, this solution has a pH of 7.4. After mounting, the PSS was warmed from room temperature to 37°C and the arterial segments were allowed to equilibrate for 60 min with transmural pressure set at 50 mmHg by raising reservoir bottles connected to the micropipettes. A pressure transducer between the micropipettes and the reservoir bottles was used to monitor transmural pressure across the arterial segment. The vessels were magnified with an inverted microscope equipped with a video camera and a monitor. Arterial diameter was measured using a video scaler calibrated with an optical micrometer. Before testing for myogenic responses to changes in transmural pressure, contractility and endothelial function were tested using 30 mM K^+ or ACh (10^{-5} M), respectively. Myogenic responses were tested by increasing or decreasing luminal pressure in 20 mmHg increments with a 5-min equilibration period at each pressure level before diameter measurements are made. After the initial myogenic responses were evaluated, the PSS was replaced with calcium-free PSS and myogenic responses were assessed again.

Rats were assigned to one of the following three groups (n=5 rats per group):
Sham-TBI - MCA's were harvested 5 minutes after sham injury.
TBI - MCA's were harvested 5 minutes after moderate, central fluid percussion TBI.
TBI-arg - Rats were treated with L-arginine (100mg/kg) before TBI and then MCA's were harvested 5 minutes after TBI.

Sham-injured rats exhibited normal vasodilatory responses to reductions in transmural pressure (Figure 5). After TBI, MCA diameters decreased progressively with each reduction in transmural pressure. Rats treated with L-arginine showed less reduction in MCA inner diameters but the responses in arteries from the treated rats were not significantly different than responses in arteries harvested from untreated rats. These studies, indicating that L-arginine does not preserve myogenic responses after TBI, are consistent with previous reports indicating that CBF autoregulation is not affected by NOS inhibitors in rats [14].

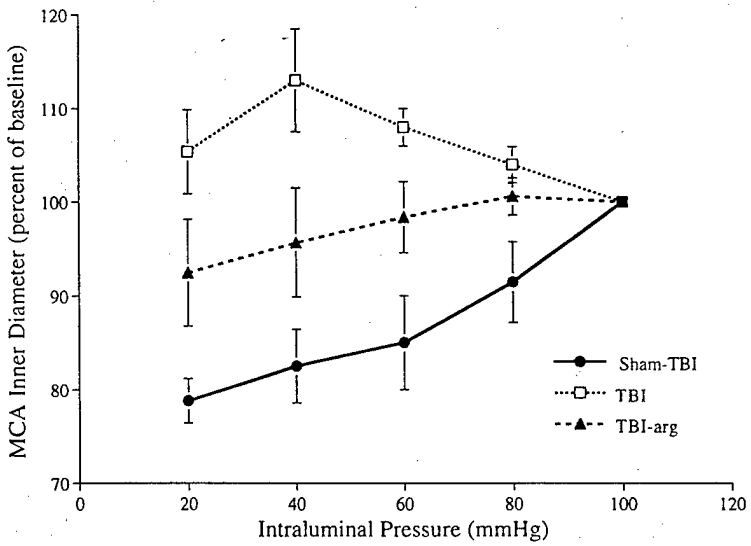

Figure 5 - Inner diameters in MCA's harvested from rats after sham-injury (Sham) or moderate TBI (TBI) or TBI and treatment with L-arginine (100mg/kg).

SA 2.2 - Microdialysis measurement of cerebral nitric oxide levels.

In order to determine whether TBI reduces cerebral NO levels, cerebral microdialysis is being used to harvest samples that will be analyzed for NO_3, a stable metabolite of NO, using an Antek 7020 NO Detector.

Rats (n=4) were anesthetized, intubated. mechanically ventilated and maintained on 1.5-2.0% isoflurane. Cannulae were placed in femoral arteries and veins as described above. The rats were then placed in a stereotaxic head holder and a midline scalp incision made. A

craniotomy was made to the right of the sagittal suture between lambda and bregma and the dura was nicked with a 25 gauge needle. The microdialysis probe was prepared according to the manufacturers instructions and recovery rate was determined using a 5% dextrose solution at 37°C. The probe was then positioned 3.6 mm posterior and 1.5 lateral to the intersection of bregma and the sagittal suture and inserted to a depth of 4 mm. The perfusion rate was set at 2μL/min and the probe was allowed to equilibrate for 30 minutes. Samples were then collected every 20 minutes for 200 minutes. Temporalis temperature, arterial blood pressure and blood gases and pH were maintained within normal limits during the sampling period. Samples were collected and analyzed on the same day using an Antek 7020 NO Detector with an Antek Model 745 NO_2/NO_3 reduction assembly and a Model 742 Data Handling Software.

Nitrate levels were somewhat elevated 20 minutes after the equilibration period but then decreased to reach a stable level which was maintained for the 200 minute sampling period (Figure 6). These studies indicate that microdialysis yields stable levels of the NO metabolite, nitrate, which will be used to monitor changes in NO levels after TBI with and without treatment with L-arginine to determine whether the effects of L-arginine on CBF after TBI are mediated by changes in NO.

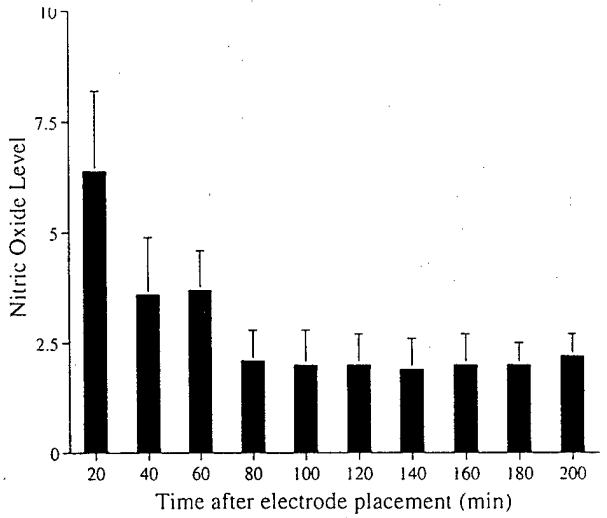

Figure 6 - Nitrate levels in the rat cerebral cortex measured using microdialysis.

SA 2.3 - Effects of peroxynitrite on myogenic responses in isolated, pressurized middle cerebral arteries.

We have reported that TBI reduces CBF in rats and that L-arginine can prevent post-TBI hypoperfusion or superoxide dismutase (SOD) can restore CBF to baseline [15] and that superoxide anion radicals are produced by TBI [16]. These results suggest that TBI may be producing superoxide radicals which destroy NO but it is also possible that superoxide is not simply destroying NO but converting it to another vasoactive agent such as peroxynitrite. Superoxide reacts with NO to produce the peroxynitrite anion ($ONOO^-$), a powerful oxidizing agent [17] which, when protonated, homiletically decays to form the highly reactive hydroxy-type radical. Peroxynitrite, in its anionic form, is remarkably stable which contributes to its toxicity by allowing it to diffuse farther from its site of formation to attack more distant cellular targets [18]. In the absence of L-arginine, NOS is capable of producing O_2^- [19]. Therefore, O_2^--mediated destruction of NO could lead to the production of additional O_2^- via NOS and L-arginine treatment may produce NO directly and, by converting NOS from O_2^- synthesis back to NO synthesis, reduce the amount of O_2^- available to inactivate NO. In addition to vasoconstriction due to reductions in NO concentrations, ONOO produced by TBI may damage the cerebral circulation and reduce compensatory responses such as autoregulation. In order to test the hypothesis that ONOO may contribute to reduced vasodilatory responses to hypotension, MCA's were harvested from uninjured rats (n=6) as described above. MCA diameters were measured as intramural pressure was reduced sequentially from 100 mmHg to 20 mmHg in 20 mmHg increments. Intramural

pressure was then returned to 100 mmHg and 25 μM ONOO was added to the bath. Intramural pressure was then sequentially reduced from 100 to 20 mmHg and arterial diameters were recorded at each 20 mmHg increment. Transmural pressure was then returned to 100 mmHg and MCA's diameters were measured in response to serotonin and acetylcholine. 25 μM ONOO had no effect on vasoconstriction to serotonin or vasodilation to acetylcholine but did abolish vasodilatory responses to hypotension (Figure 7, next page).

These studies, which are the first to demonstrate that ONOO markedly reduces vasodilatory responses to progressive hypotension in isolated MCA's, suggest that TBI may impair autoregulatory vasodilatory responses via the production of ONOO from NO and superoxide.

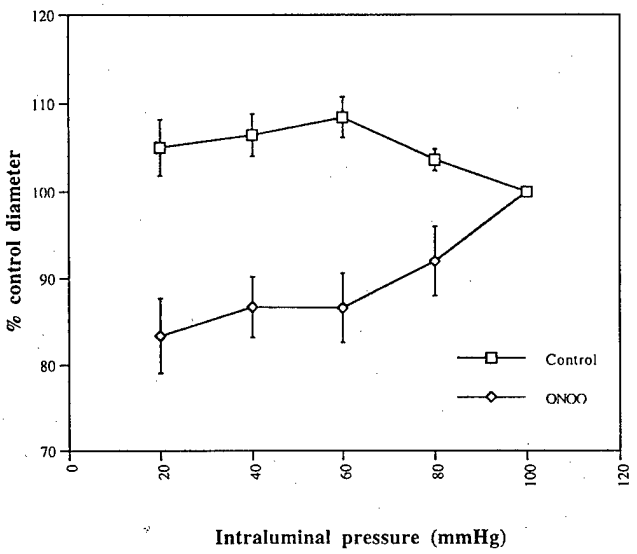

Figure 7 - Arterial diameters in rats MCA's exposed to 25 μM ONOO.

SA 2.4 - Effects of TBI on CGRP-containing perivascular nerve density.

Inhibition of CGRP receptors has been reported to reduce autoregulatory responses to hypotension in rodents. We tested the hypothesis that TBI impairs cerebral autoregulation by reducing CGRP-containing perivascular nerve density. In order to test this hypothesis,we determined whether TBI will reduce the number of CGRP-containing perivascular nerve fibers which were measured using immunohistochemical staining of CGRP and stereologic counting.

Using a protocol approved by the IACUC of The University of Texas Medical Branch, male Sprague-Dawley rats (450-550 g) were anesthetized, intubated, ventilated on 1.5-2.0% isoflurane in air:O2 (70:30), and prepared for midline fluid percussion TBI as previously described [10]. Cannulae were placed in a femoral artery and vein for monitoring of arterial blood pressure and drug infusion, respectively. The isoflurane was lowered to 1.5% and rats were randomly chosen to receive a sham TBI or moderate (2.0 atm) or severe (3.0 atm.) TBI. Following the TBI, isoflurane was increased to 2.0% and the rats were perfused transcardially with normal saline to remove the blood. The cerebral arterial circle of Willis and its large branches were removed and stretched flat on gelatin-treated microscope slides and fixed for 10 min in ice cold methanol. After rinsing in Tris buffered saline, intrinsic peroxidase activity was blocked using a commercial peroxidase blocker (DAKO, Inc.) and non-specific protein binding was blocked by incubation with a serum-free blocker (DAKO, Inc.). The slides were then incubated at 4°C overnight with polyclonal anti-CGRP (1:500) and then with the biotin-conjugated secondary antibody. The antigen-antibody complex was visualized using commercially available stains, dehydrated and permanently mounted. Nerve density will be determined using stereologic methods [20]. Briefly, analysis of all the CGRP-containing nerves in each section was made using photomicrographs of the vessel segment taken at different planes of focus. Intersections of nerves with a plastic grid

were then counted by a blinded observer and the density as length/area will be calculated at $\pi N/2L$, where N is the number of intersections and L is the total grid length.

There was no significant difference in CGRP-containing perivascular nerve density in rats after low or high levels of central fluid percussion injury (Figure 8). These studies suggest that CGRP containing perivascular nerves are not affected by TBI and, therefore, are probably not involved in TBI-induced impairment of autoregulation However, it is possible that TBI affects other vasodilatory agents that may be contained in perivascular nerves.

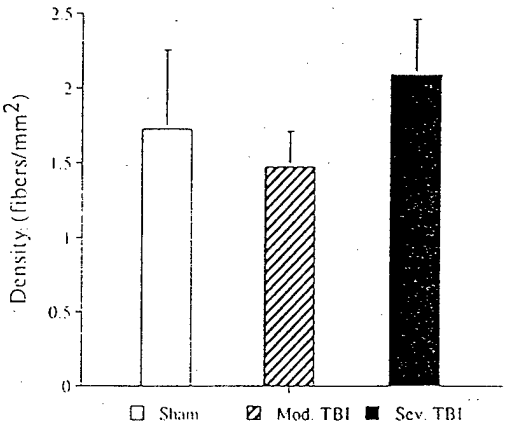

Figure 8 - Density (fibers/mm2) of CGRP immunoreactive perivascular nerve fibers harvested from rats after sham-TBI (Sham) or moderate (Mod. TBI) or severe (Sev. TBI) TBI. values are mean ± sem.

SA 2.5 Myogenic responses to hypotension: Possible role of anandamide and perivascular nerves.

In addition to these known perivascular neurotransmitters, other agents have been reported to be cerebral vasodilators. One such agent is anandamide, an endogenous ligand of the cannabinoid 1 (CB1) receptor. Anandamide is a potent cerebral vasodilator [21] and we have exciting new evidence that inhibition of the anandamide receptor reduces myogenic responses in isolated pressurized cerebral arteries. Rats were anesthetized with isoflurane, decapitated and their MCA's were harvested, mounted in the arteriograph and allowed to equilibrate as described above. Changes in MCA diameters were measured as intraluminal pressure was reduced progressively from 100 to 20 mmHg in 20 mmHg increments. In the first group of rats (n=5) myogenic responses were tested twice with approximately 30 min. in between tests. In this time control group, myogenic responses were similar between the first and second test (data not shown). As second group of rats (n=12) was treated identically except that the vessel were filled with SR141716A, a specific antagonists of the CB1 receptor (3μM, n=6) or with the ethanol vehicle (n=6). In this group, the ethanol vehicle had no effect on myogenic responses (data not shown) but myogenic responses were reduced significantly compared to the pre-treatment responses in the SR141716A-treated arteries (Figure 9).

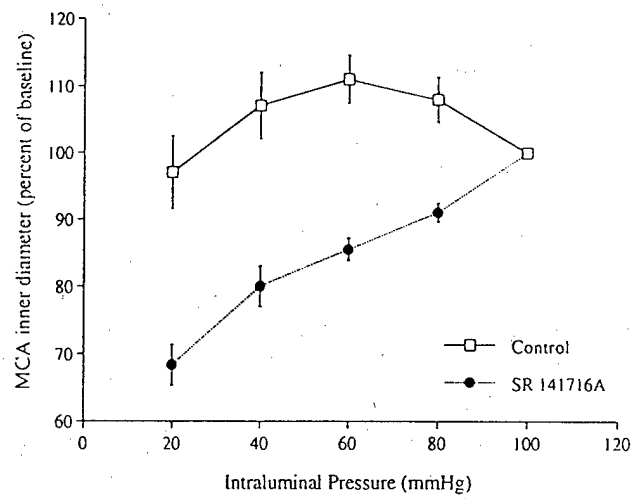

Figure 9 - Inner diameters in MCA's before (Control) and after (SR141716A) treatment with SR141716A (3μM).

A fourth group of rats was prepared identically to the other three groups but were treated with extraluminal application of phenol (0.5%) for 10 minutes in between the two evaluations of myogenic responses. Phenol treatment did not affect vasoconstrictor responses to serotonin but significantly reduced myogenic responses to progressive reductions in intraluminal pressure (Figure 10). Phenol, when applied at the concentrations and times used in this study, destroys adventitial perivascular nerve fibers without affecting the underlying vascular smooth or endothelium.

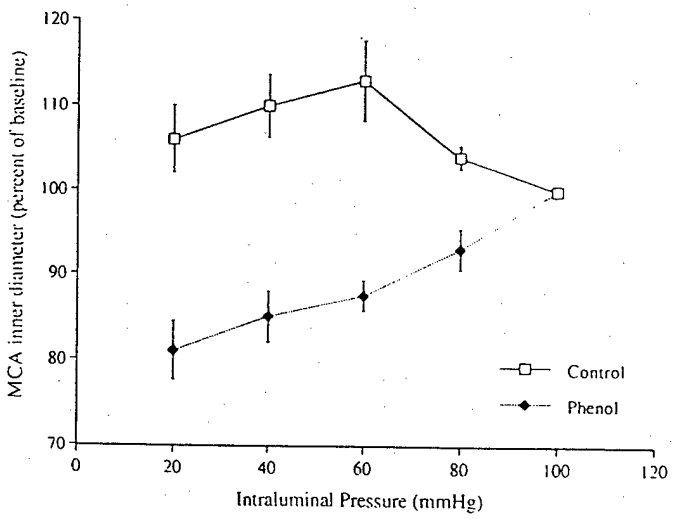

Figure 10 - Inner diameters in MCA's before (Control) and after (Phenol) extraluminal treatment with phenol (0.5% for 10 min).

These studies suggest that perivascular nerve fibers may be involved in vasodilatory responses to hypotension, perhaps by the action of an endogenous cannabanoid ligand such as anandamide.

Specific Aim 3 is to address the hypothesis that increased production of O_2^- during TBI and subsequent hypotension/resuscitation is responsible for the impaired cerebrovascular reactivity.

Hypothermia is known to be protective in TBI and cerebral ischemia but the mechanisms are not well understood. Hypothermia reduces damage due to oxygen free radicals in a variety of situations [22,23] and oxygen radical scavengers restore cerebral vascular reactivity after TBI [9]. Therefore, we hypothesized that hypothermia might be neuroprotective after TBI. These studies are related to SA 3 because findings that preserved autoregulation by hypothermia, which is known to reduce oxidative damage, would support that underlying hypothesis of SA 3. In fact, we found that hypothermia did not preserve autoregulation suggesting that hypothermia does not reduce O_2^- after TBI or that O_2^- does not contribute to impaired cerebral vascular reactivity after TBI.

SA 3.1 - Effects of hypothermia on CBF after TBI:

Mild hypotension worsens outcome after TBI in humans [24], perhaps because of TBI-induced impaired cerebral vasodilation [25]. Hypothermia has improved outcome after TBI in animals [26] and humans [27] but the mechanism remains unclear. This study evaluated the effects of hypothermia on CBF pressure autoregulation after fluid percussion injury (FPI) in rats.

In an approved protocol, 31 Sprague-Dawley rats were anesthetized with isoflurane, intubated, mechanically ventilated with 1.5-2% isoflurane in 2:1 air:oxygen and prepared for right lateral fluid percussion TBI and cerebral laser Doppler flowmetry (LDF). A 4.8mm trephine craniotomy was created midway between the sagittal suture and the insertion of the temporalis muscle and midway between lambda and bregma followed by insertion of machined TBI adapter. The skull was thinned with an air-cooled dental drill on the left midway between the sagittal suture and the insertion of the temporalis muscle and midway between lambda and bregma for placement of the LDF probe. The animal was turned supine and via a mid-line abdominal incision, the aorta exposed at the level of the iliac bifurcation, cannulated with PE210 tubing, heparinized with 50 units heparin, and connected via silastic tubing to a large volume variable height reservoir. The reservoir was primed with 20ml of donor rat whole blood (.5cc CPD solution and 12.5 units heparin for each 10cc whole blood) and a timer begun. The animal was placed prone in a stereotaxic head holder, the FPI device attached via a 60cm length of non-compliant tubing, the LDF probe placed, and bilateral temporalis muscle temperature probes attached. Temperature was maintained at 37 ± 0.5 37° C using heating lamps, heating blanket, fans, and iced isopropyl alcohol as needed. Isoflurane was decreased to 1%, and baseline arterial blood gas and hematocrit obtained. Mechanical ventilation was adjusted to maintain a $PaCO_2$ of 33-38mmHg without correction for temperature (alpha-stat management) throughout the study. CBF autoregulation was obtained by measuring changes in LDF at various arterial blood pressures. The systemic arterial blood pressure was measured at the junction of the aorta and the silastic tubing, while the blood pressure was manipulated by raising and lowering the reservoir height with respect to the animal. LDF was measured at a mean arterial pressure (MAP) of 100 mmHg (used as the baseline blood pressure and CBF) followed by randomly assigned changes in blood pressure to means of 80, 60, or 40 mmHg. After stabilization of the blood pressure and LDF signal at each blood pressure (120 to 240 seconds) the MAP and CBF were recorded and the blood pressure returned to 100 mmHg for a 2-3 minute restabilization period. The process was repeated until all pressure perturbations were completed. After baseline autoregulation testing at 37° C, the animals were randomized to one of 5 groups:

37° C without FPI (Control 37)
37° C with FPI of 1.8 atm (FPI 37)
32° C without FPI (Control 32)
32° C with FPI 1.8 atm (Pre-FPI 32)
37° C with FPI 1.8 atm and immediate cooling to 32° C (Post-FPI 32).

After collecting ABG and hematocrits, the correct temperature was established and the appropriate FPI delivered. Cerebral autoregulation measurement was repeated at 30 and 60 minutes following FPI. At the completion of the study, the animals were killed with saturated KCl. Individual animal

exclusion criteria included: study time greater than 120 minutes from aortic occlusion to study completion, hematocrit less than 34, pH of < 7.3 with $PaCO_2$ in the target range, PaO_2 < 100 mmHg, or death of the animal during the preparation.

There were no significant differences among groups with respect to animal weight, $PaCO_2$, pH, PaO_2 or hematocrit. At baseline, no significant differences in CBF were observed among groups at any level of MAP (Figure 11). CBF changes occurred in the TBI and TBI plus hypothermia groups at 30 and 60 minutes (Figures 12 & 13). There were no significant differences in CBF changes between the normothermic TBI (FPI 37) and the TBI plus hypothermia (Pre-FPI 32 and Post-FPI 32) groups. These studies indicate that hypothermia initiated prior to or following TBI does not lead to improvements in CBF when compared to normothermic TBI. Therefore, the protective mechanisms of hypothermia are most likely related to mechanisms other than the restoration of CBF after TBI.

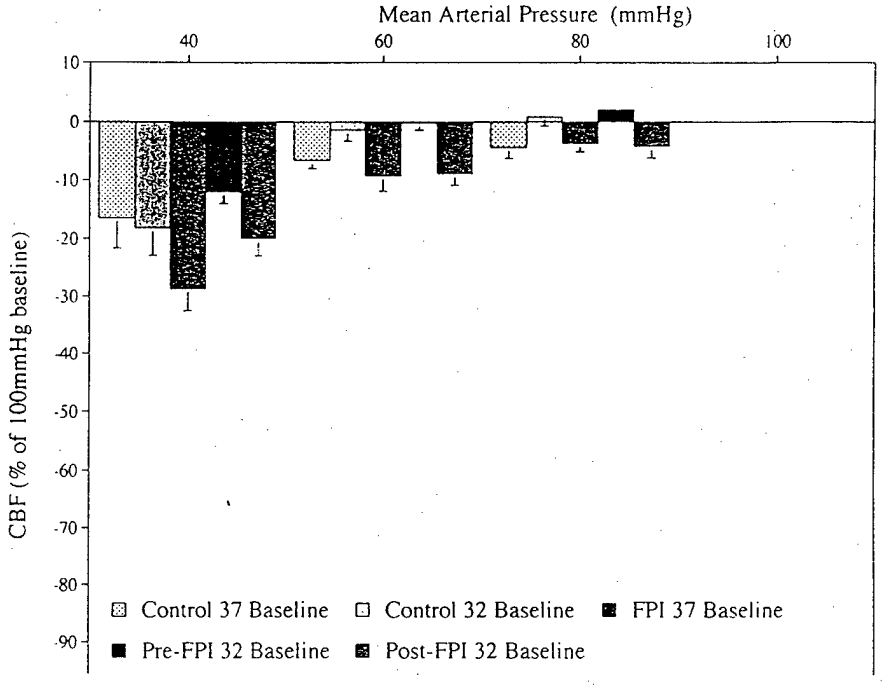

Figure 11 - Comparison of percent changes in CBF (compared to 100 mmHg baseline) across groups at prior to TBI.

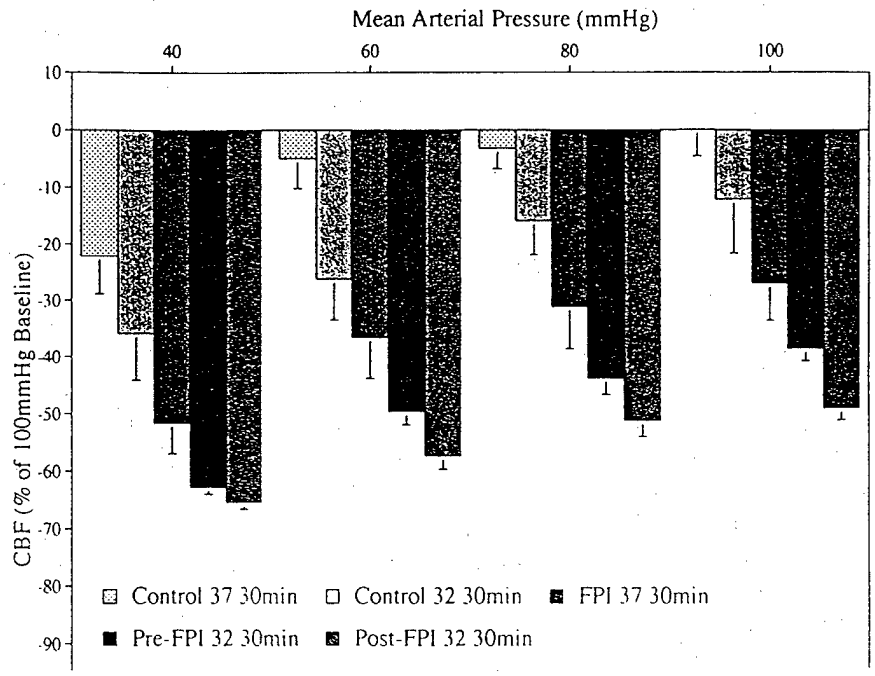

Figure 12 - Comparison of percent changes in CBF (compared to 100 mmHg baseline) across groups 30 minutes after TBI.

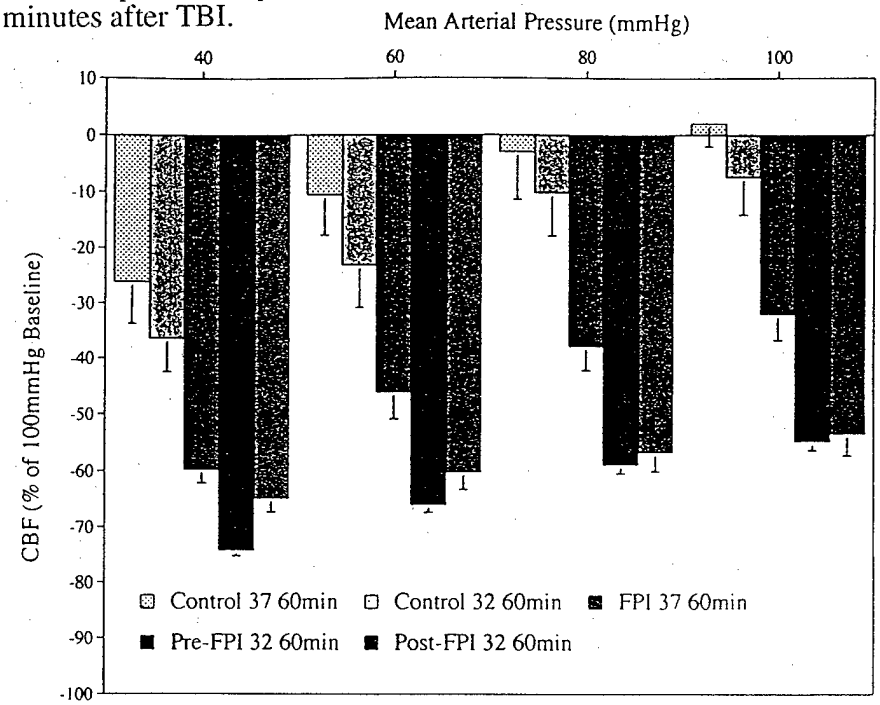

Figure 13 - Comparison of percent changes in CBF (compared to 100 mmHg baseline) across groups 60 minutes after TBI.

Specific Aim 4 will address the hypothesis that small volume resuscitation with hypertonic saline will restore cerebral circulatory and systemic hemodynamics without causing the pronounced changes in brain water diffusion seen after TBI and hypotension/resuscitation with shed blood.

SA 4.1 - Effects of hypertonic solutions on CBF and ICP after TBI and hypotension.

This application is based on a series of exciting observations that L-arginine, the precursor of the endothelium dependent vasodilator, nitric oxide (NO), completely prevents the hypoperfusion that occurs after experimental traumatic brain injury (TBI) in rats [10]. We have made

direct simultaneous measurements of cortical blood flow and O_2^- levels after TBI in rats [1016] that suggest that increases in O_2^- levels immediately after TBI initiate a process that results in hypoperfusion after TBI. We have demonstrated that treatment with the NOS substrate L-arginine prevented CBF reductions and that SOD restored CBF after TBI, suggesting that TBI-generated oxygen radicals inactivate NO or NOS and reduce CBF [10]. Further studies indicated that TBI does not affect NOS activity directly [7] suggesting that TBI directly inactivates NO rather than affecting NOS activity. What remains to be determined is whether L-arginine effectively restores CBF after TBI and hemorrhagic hypotension and which dose of L-arginine in hypertonic saline is most effective.

Rats were prepared for TBI and measurement of CBF with LDF as described above. Arterial blood pressure and CBF were measured in rats treated with one of three doses of L-arginine (50, 100mg/kg or 300mg/kg, i.v., n = 7 per group) in hypertonic solutions (2400 mOsm total in NaCl) after TBI (moderate fluid percussion injury) and hemorrhagic hypotension (MAP=60mmHg for 45 min). CBF was measured prior to TBI (Baseline), during the hemorrhage period (Hem.), after reinfusion was one of the hypertonic arginine solutions (Inf.), 30, 60 and 120 min after reinfusion.

All three hypertonic arginine solutions improved MAP nearly to baseline immediately after reinfusion but MAP fell to 59%, 68% and 60% of baseline in the 50, 100 and 300 mg/kg L-arginine groups, respectively (Figure 14). Repeated measures analysis of variance revealed no interaction between group and MAP levels ($p < 0.267$).

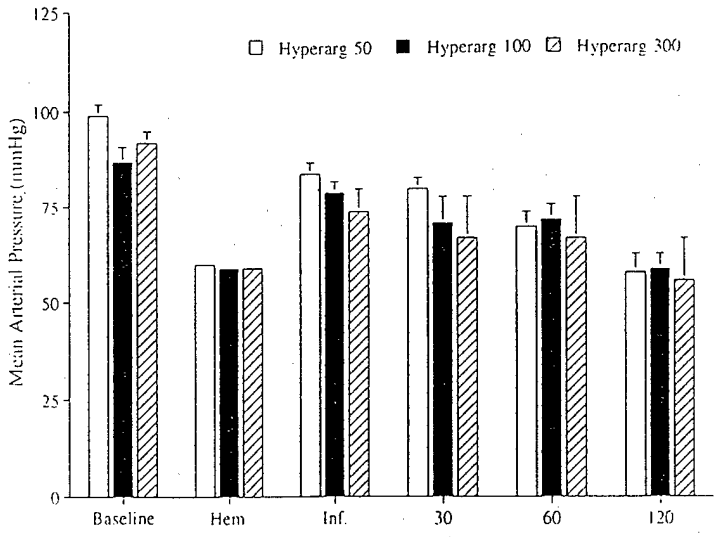

Figure 14 - Mean arterial pressure (MAP) in rats treated with 50 (Hyperarg 50), 100 (Hyperarg 100) or 300 (Hyperarg 300) mg/kg of L-arginine in hypertonic saline

Cerebral blood flow decreased to about 60% of baseline in all groups during hemorrhage after TBI (Figure 15). During reinfusion and 30 and 120 minutes after reinfusion, CBF levels were highest in the group treated with a hypertonic arginine solution containing 100 mg/kg of L-arginine. After reinfusion, CBF in the Hyperarg 100 group was 86% of baseline compared to 68% and 54% of baseline in the Hyperarg 50 and Hyperarg 300 groups, respectively. Similarly, two hours after resuscitation, CBF in the Hyperarg 100 group was 77% of baseline compared to 59% and 56% of baseline in the Hyperarg 50 and Hyperarg 300 groups, respectively. Therefore, these "dose-response" studies suggest that hypertonic saline-L-arginine solutions can partially restore CBF after TBI and hemorrhage and, since CBF was 77% of baseline CBF 120 minute after reinfusion, that CBF restoration persists for hours after treatment. These studies also demonstrate that the 100mg/kg dose of arginine in hypertonic saline is the most effective concentration. In the

next funding period we will compare hypertonic arginine solutions will other standard resuscitation solutions (e.g. lactated Ringer's) to determine whether hypertonic arginine solutions may be a more effective resuscitation agent.

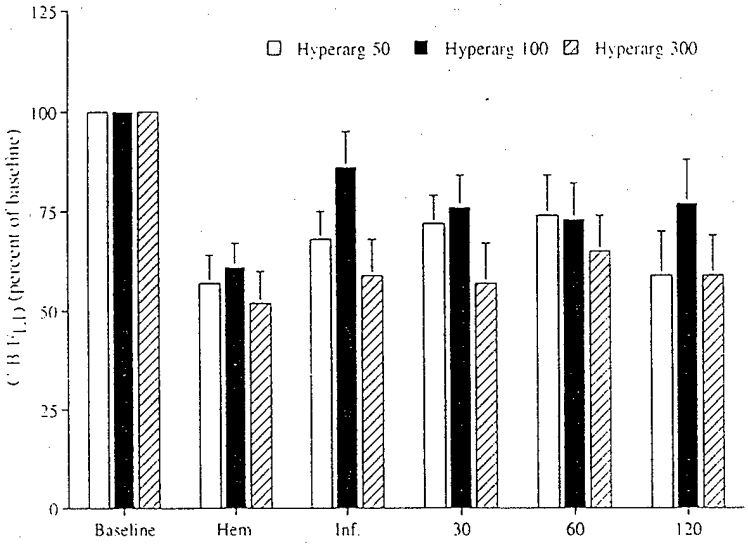

Figure 15 - Cerebral blood flow (CBF) in rats treated with 50 (Hyperarg 50), 100 (Hyperarg 100) or 300 (Hyperarg 300) mg/kg of L-arginine in hypertonic saline.

CONCLUSIONS

These studies demonstrate that significant progress towards the aim of the application have been made in the first two years. Our research has resulted in novel and important observations related to the mechanisms of traumatic vascular injury. We have reported a model that allows for sequential, accurate and quantitative measurements of regional CBF using MRI. We have used MRI to measure CBV, TT, CBF and brain and arterial AUC after TBI only, hemorrhagic hypotension only or the combined injury. We have also demonstrated that L-arginine (100 mg/kg) prevents CBF reductions after TBI but does not significantly improve myogenic responses in isolated pressurized MCA segments. We have demonstrated that we can measure cerebral NO levels using microdialysis. Furthermore, we have demonstrated that peroxynitrite can abolished compensatory vasodilatory responses to hypotension in isolated middle cerebral arteries. We have provided evidence suggesting that the potent vasodilatory transmitter, CGRP, is probably not involved in traumatic vascular injury since TBI doesn't reduce the density of CGRP containing perivascular nerve fibers. We have reported novel and exciting evidence that anandamide, an endogenous ligand of the CB1 receptor, may contribute to normal myogenic responses in the cerebral circulation. In addition, our evidence that phenol, which destroys perivascular nerves, also reduces myogenic responses to hypotension suggests that the perivascular nerves may be the source of the anandamide. These studies suggest that TBI may impair the CB1 receptor or may reduce the levels or production of anandamide by the brain or by perivascular nerves. We have shown that hypothermia, which reduces oxygen free radicals and is neuroprotective after TBI or stroke does not act by improving CBF after TBI. Finally, we have demonstrated that a new class of resuscitation solutions containing L-arginine in hypertonic saline may provide immediately and lasting improvements in CBF after TBI.
These hypotheses will be investigated further during the next year of the funding period.

The following articles, book chapter, editorials and abstracts were published or submitted during the previous funding period. Items included in Appendix 1 are marked with an asterisk.

The following articles, book chapter, editorials and abstracts were published or submitted during the previous funding period. Items included in Appendix 1 are marked with an asterisk.

1. DeWitt DS, Prough DS, Uchida T, Deal DD, Vines SM. The effects of nalmefene, CG3703, tirilazad or dopamine in cerebral blood flow, oxygen delivery, and electroencephalographic activity after traumatic brain injury and hemorrhage. J Neurotrauma 14:931-941, 1997

2. DeWitt DS, Prough DS. Accurate measurement of brain temperature. Crit Care Med 26:431-432, 1998

3. DeWitt DS, Prough DS. Vasoactive prostanoids and traumatic brain injury. Crit Care Med 26:819-821, 1998

4. Fabian RH, DeWitt DS, Kent TA. The 21-aminosteroid U-74389G reduces cerebral superoxide anion concentration following fluid percussion injury of the rat. J Neurotrauma 15:433-439, 1998

*5. Alagarsamy S, DeWitt DS, Johnson KM. Traumatic brain injury does not affect nitric oxide synthase activity in rats. J Neurotrauma 15: 625-631, 1998.

6. Bedell, DeWitt DS, Prough DS. Fentanyl infusion preserves cerebral blood flow during decreased mean arterial blood pressure following traumatic brain injury in cats. J Neurotrauma 15:985-992, 1998.

7. Hulsebosch CE, DeWitt DS, Jenkins LW, Prough DS. Traumatic brain injury in rats results in increased expression of GAP-43. Neurosci Lett 255:83-86, 1998.

8. Davis KL, Jenkins LW, DeWitt DS, Prough DS. Mild traumatic brain injury does not modify the cerebral blood flow profile of secondary forebrain ischemia in Wistar rats. J Neurotrauma 15:615-625, 1998

9. DeWitt DS, Mathew B, Jenkins LW, Prough DS. Traumatic brain injury reduces myogenic responses to hypotension in isolated middle cerebral arteries from rats. Anesthesiology 89:A694, 1998

10. Conroy BP, Jenkins LW, Grafe M, DeWitt DS, Johnston WE. Hypothermic neuroprotection during hyperglycemic cardiopulmonary bypass in pigs. Anesthesiology 89:A693, 1998

11. Deyo DJ, DeWitt DS, Miller KR, Prough DS. Development of a balloon-expansion model of epidural hematoma in the rat. J Neurotrauma 15:866, 1998

12. Bedell EA, Williams JP, DeWitt DS, Prough DS. The influence of transcranial conduit length on gross morphologic brain injury after parasagittal fluid percussion injury in rats. J Neurotrauma 15:858, 1998

13. DeWitt DS, Mathew B, Jenkins LW, Prough DS. Traumatic brain injury impairs myogenic responses in rodent middle cerebral arteries. J Neurotrauma 15:866, 1998

14. Wang CZ, Parsley MA, Jenkins LW, Johnson KM, DeWitt DS, Prough DS. Hypothermia

16. DeWitt DS, Prough DS. Improving cerebral perfusion after traumatic brain injury. Crit. Care Med. 27:(in press), 1999

17. DeWitt DS, Prough DS, Deal DD, Vines SM, Uchida T. Inhibition of nitric oxide synthase does not reduce cerebral vascular responses to hemodilution or hemorrhagic hypotension in rats. Amer. J. Physiol. (in review).

18. Mathew B, DeWitt DS, Bryan RM, Bukoski RD, Prough DS. Traumatic brain injury reduces myogenic responses in pressurized rodent middle cerebral arteries. J Neurotrauma (in review).

REFERENCES CITED

1. Hong KW, Pyo KM, Lee WS, Yu SS, Rhim BY. Pharmacological evidence that calcitonin gene-related peptide is implicated in cerebral autoregulation. *American Journal of Physiology* 1994;266:H11-H16.

2. Axel L. Cerebral blood flow determination by rapid-sequence computed tomography. *Radiology* 1980;137:679-686.

3. Mottet I, Quast MJ, DeWitt DS et al. N G -Nitro-L-Arginine methyl ester modifies the input funciton measured by dynamic susceptibility contrast magnetic resonance imaging. 1997;17:791-800.

4. Ostergaard L, Smith DF, Vestergaard-Poulsen P et al. Absolute cerebral blood flow and blood volume measured by magnetic resonance imaging bolus tracking: Comparison with positron emission tomography values. *J. Cereb. Blood Flow Metab.* 1998;18:425-432.

5. Yamakami I, McIntosh TK. Effects of traumatic brain injury on regional cerebral blood flow in rats as measured with radiolabeled microspheres. 1989;9:117-124.

6. DeWitt DS, Alagarsamy S, Johnson KM, Prough DS. Traumatic brain injury does not reduce total or stimulated nitric oxide synthase activity. *J Cereb Blood Flow Metab* 1997;17(Suppl 1):S17.

7. Alagarsamy S, DeWitt DS, Johnson KM. Effects of moderate, central fluid percussion traumatic brain injury of nitric oxide synthase activity in rats. *J. Neurotrauma* 1998;15:627-633.

8. DeWitt DS, Prough DS, Taylor CL, Whitley JM, Deal DD, Vines SM. Regional cerebrovascular responses to progressive hypotension after trauamtic brain injury in cats. 1992;263:H1276-H1284.

9. Wei EP, Kontos HA, Dietrich WD, Povlishock JT, Ellis EF. Inhibition by free radical scavengers and by cyclooxygenase inhibitors of pial arteriolar abnormalities from concussive brain injury in cats. *Circulation Research* 1981;48:95-103.

10. DeWitt DS, Smith TG, Deyo DJ, Miller KR, Uchida T, Prough DS. Posttraumatic hypoperfusion in rats is prevented or reversed by treatment with L-arginine or superoxide dismutase. *J Neurotrauma* 1997;14:223-233.

11. Mathew B, DeWitt DS, Bryan RM, Bukoski RD, Prough DS. Traumatic brain injury reduces myogenic responses in pressurized rodent middle cerebral arteries. *J. Neurotrauma* 1999;16:in press.

12. Dixon CE, Lyeth BG, Povlishock JT et al. A fluid percussion model of experimental brain injury in the rat. *Journal of Neurosurgery* 1987;67:110-119.

13. Bryan RM, Eichler MY, Swalford MWG, Johnson TD, Suresh MS, Childres WF. Stimulation of α_2 adrenergic adrenorecptors dilates the rat middle cerebral artery. *Anesthesiology* 1996;85:82-90.

14. Wang Q, Paulson OB, Lassen NA. Is autoregulation of cerebral blood flow in rats influenced by nitro-L-arginine, a blocker of the synthesis of nitric oxide? *Acta Physiologica*

Scandinavia 1992;145:297-298.

15. DeWitt DS, Smith TG, Deyo DJ, Miller KR, Uchida T, Prough DS. L-arginine and superoxide dismutase prevent or reverse cerebral hypoperfusion after fluid-percussion traumatic brain injury. 1997;14:219-229.

16. Fabian RH, DeWitt DS, Kent TA. In vivo detection of superoxide anion production by the brain using a cytochrome c electrode. 1995;15:242-247.

17. Beckman JS. The double-edged role of nitric oxide in brain function and superoxide-mediated injury. *Journal of Developmental Physiology* 1991;15:53-59.

18. Beckman JS, Chen J, Ischiropoulos H, Crow JP. Oxidative chemistry of peroxynitrite. *Methods in Enzymology* 1994;233:229-240.

19. Pou S, Pou WS, Bredt DS, Snyder SH, Rosen GM. Generation of superoxide by purified brain nitric oxide synthase. *The Journal of Biological Chemistry* 1992;267:24173-24176.

20. Schmid-Schoenbein GW, Zweifach BW, Kovalcheck S. The application of sterological principles to morphometry of the microcirculation in different tissues. *Microvascular Research* 1977;14:303-317.

21. Ellis EF, Moore SF, Willoughby KA. Anandamide and delta9-THC dilation of cerebral arterioles is blocked by indomethacin. *American Journal of Physiology* 1995;269:H1859-H1864.

22. Globus M, Alonso O, Dietrich W, Busto R, Ginsberg M. Glutamate release and free radical production following brain injury: effects of posttraumatic hypothermia. *J Neurochem* 1995;65(4):1704-1711.

23. Zhao W, Richardson JS, Mombourquette MJ, Weil JA, Ijaz S, Shuaib A. Neuroprotective effects of hypothermia and U-78517F in cerebral ischemia are due to reducing oxygen-based free radicals: an electron paramagnetic resonance study with gerbils. *Journal of Neuroscience* 1996;45:282-288.

24. Chesnut RM, Marshall LF, Klauber MR et al. The role of secondary brain injury in determining outcome from severe head injury. 1993;34:216-222.

25. DeWitt DS, Prough DS, Taylor CL, Whitley JM, Deal DD, Vines SM. Regional cerebrovascular responses to progressive hypotension after traumatic brain injury in cats. *American Journal of Physiology* 1992;263:H1276-H1284.

26. Clifton GL, Jiang JY, Lyeth BG, Jenkins LW, Hayes RL. Marked protection by moderate hypothermia after experimental traumatic brain injury. 1991;11:114-121.

27. Marion DW, Penrod LE, Kelsey SF et al. Treatment of traumatic brain injury with moderate hypothermia. 1997;336:540-546.

APPENDIX

The appendix consists of a reprint of the following article:

Alagarsamy S, DeWitt DS, Johnson KM. Traumatic brain injury does not affect nitric oxide synthase activity in rats. J Neurotrauma 15: 625-631, 1998.

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Volume 15, Number 8, 1998
Mary Ann Liebert, Inc.

Effects of Moderate, Central Fluid Percussion Traumatic Brain Injury on Nitric Oxide Synthase Activity in Rats

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ABSTRACT

Experimental traumatic brain injury (TBI) damages cerebral vascular endothelium and reduces cerebral blood flow (CBF). The nitric oxide synthase (NOS) substrate, L-arginine, prevents CBF reductions after TBI, but the mechanism is not known. This study examined the possibility that posttraumatic hypoperfusion is due to reductions in the substrate sensitivity of NOS which are overcome by L-arginine. Isoflurane-anesthetized rats were prepared for TBI (midline fluid-percussion, 2.2 atm), sham-TBI, or no surgery (control), and were decapitated 30 min after injury or sham injury. The brains were removed and homogenized or minced for measurements of crude soluble or cell-dependent stimulated NOS activity, respectively. Baseline arterial oxygen, carbon dioxide, pH, or hemoglobin levels did not differ among control, sham, or TBI groups. Total cortical soluble NOS activity in TBI-treated rats was not significantly different from either untreated or sham groups when 0.45 μM or 1.5 μM L-arginine was added. Also, there were no differences in cell-dependent NOS activity among the three groups stimulated by 300 μM *N*-methyl-D-aspartate, 50 mM K^+, or 10 μM ionomycin. These data suggest that TBI reduces CBF by a mechanism other than altering the substrate specificity or activation of nNOS.

Key words: arginine; calcium; cerebral blood flow; cortex; fluid percussion; glutamate

INTRODUCTION

TRAUMATIC BRAIN INJURY (TBI) results in reduced cerebral blood flow (CBF) in the first few hours after injury (Bouma et al., 1991, 1992). Although the role of posttraumatic hypoperfusion in pathophysiology is not known, evidence of ischemia in most TBI patients (Graham et al., 1978) suggests that CBF reductions may be important contributors. Early posttraumatic hypoperfusion occurs after experimental TBI (Yuan et al., 1988; Yamakami and McIntosh, 1989, 1991). The causes of significant reductions in CBF after TBI in patients or experimental animals are not known but posttraumatic hypoperfusion may result from impairment or destruction of a cerebral vasodilatory mechanism. The endothelium-dependent relaxing factor, nitric oxide (NO), is one such cerebral vasodilator (Furchgott and Zawadzki, 1980; Ignarro et al., 1987). Evidence that inhibition of NO synthesis decreases CBF (Beckman et al., 1991; Tanaka et al., 1991; DeWitt et al., 1992; Pelligrino et al., 1993) suggests a resting cerebral vasodilatory tone owing to the continuous production of NO. Nitric oxide is a free radical that is inactivated (Rubanyi and Vanhoutte, 1986) or converted to an even more reactive species such as the peroxynitrite anion (Beckman, 1991; Beckman et al., 1994; Crow and Beckman, 1995) by oxygen free radicals

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such as superoxide. Superoxide anion radicals are produced by fluid-percussion TBI (Wei et al., 1981; Fabian et al., 1995), perhaps as a byproduct of trauma-induced increases in prostaglandin synthesis (DeWitt et al., 1988). Free radicals contribute to the pathophysiology of TBI because the cyclooxygenase inhibitor indomethacin or the free radical scavenger superoxide dismutase reduces impaired cerebral vascular reactivity and endothelial damage after TBI (Wei et al., 1981). Recent evidence that treatment with L-arginine or superoxide dismutase improves CBF after experimental TBI (DeWitt et al., 1997) supports the hypothesis that radical-mediated inactivation of NO or nitric oxide synthase (NOS) contributes to posttraumatic hypoperfusion. Whether TBI affects NO levels directly or reduces NO by affecting NOS remains unclear. To determine whether NOS activity is altered by brain trauma, the conversion of L-arginine to citrulline was measured in rats subjected to moderate central fluid-percussion TBI.

MATERIALS AND METHODS

Surgical Preparation

All experimental protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas Medical Branch. Male Sprague-Dawley rats weighing 350–400 g were anesthetized with isoflurane in an anesthetic chamber, intubated, and mechanically ventilated with 1.5–2.0% isoflurane in O_2/room air (70:30) using a volume ventilator (EDCO Scientific, Chapel Hill, NC). Polyethylene cannulae were placed a femoral artery and vein for drug infusion and arterial pressure monitoring, respectively. Rectal temperature was monitored using a telethermometer (Yellow Springs Instruments, Yellow Springs, OH) and maintained using a thermostatically controlled water blanket (Gaymar, Orchard Park, NY). Rats were prepared for midline fluid-percussion TBI as previously described (Dixon et al., 1987). Briefly, the rats were placed in a stereotaxic frame and the scalp was sagitally incised. A 4-mm hole was trephined into the skull over the sagittal suture approximately midway between lambda and bregma and a modified Luer-Lok syringe hub was placed over the exposed dura and bonded in place with cyanoacrylic adhesive and covered with dental acylic. Isoflurane was decreased to 1.5%; the rats were connected to the trauma device and subjected to moderate (2.2 atm) TBI. The animals in the sham group were subject to the same surgical procedure as the TBI group with the exception of the actual insult. Brains were harvested 30 min after surgery (control group did not have surgery), TBI, or sham-TBI, and the cortices were used for cell-independent and cell-dependent biochemical assays as described below. All assays were performed blinded as to experimental group.

Experimental Design

All rats were prepared for sham injury or fluid-percussion TBI as described above. Control rats were decapitated without surgical preparation. To measure total baseline soluble NOS activity, control rats ($n = 10$), or rats subjected to sham injury ($n = 5$) or moderate (2.2 atm), central, fluid percussion TBI ($n = 5$) were decapitated approximately 30 min after injury or sham-injury. Brains were removed, the hemispheres were separated from the brain stem and prepared for crude enzyme assessments of total soluble NOS activity as described below. Saturating concentrations of cofactors and sufficient amounts of substrate were combined with NOS inhibitors when appropriate. The inhibitors used in these experiments were 3-bromo-7-nitroindazole (30 μM) and *S*-methyl-thiocitrulline (10 μM).

To measure cell-dependent NOS activity, control rats ($n = 9$) or rats subjected to sham-injury ($n = 5$) or moderate (2.2 atm) central fluid-percussion TBI ($n = 5$) were decapitated approximately 30 min after injury or sham injury. Brains were removed and mince preparations were prepared for assessment of NOS activity in response to stimulation with 300 μM NMDA, 50 mM potassium chloride, or 10 μM ionomycin.

In both studies, statistical differences were determined by analysis of variance followed by Dennett's test where appropriate. A p value of <0.05 was considered significant.

Cell-Independent NOS Activity

Measurements of soluble NOS activity in crude enzyme preparations based on methods of Bredt and Snyder (1989) were made by obtaining and homogenizing the cerebral cortex in three volumes buffer containing 0.32 M sucrose, 20 mM HEPES, 0.5 mM EDTA, and 1 mM dithiothretol. This homogenate was centrifuged for 5 min at 3,000*g*. The supernatant was spun at 20,000*g* for 15 min. The supernatant from the second spin was passed over a Dowex AG50WX-8 ion exchange column to remove the endogenous arginine. Activity of the soluble NOS enzyme was monitored by adding Ca^{2+} (0.9 mM), NADPH (10 mM), and [^{3}H]arginine (50 mM) (Amersham, Arlington Heights, IL) to the homogenate and measuring the levels of [^{3}H]citrulline produced in a 20-min incubation at room temperature. In a group of rats, the NOS inhibitors 3-bromo-7-nitroindazole or *S*-methyl-thiocitrulline were added during this incubation period. The [^{3}H]arginine was separated from the [^{3}H]citrulline by ion exchange chromatography as described be-

low. These data are presented as amount of [^{3}H]citrulline divided by the amount of [^{3}H]arginine + [^{3}H[citrulline × 100/mg of protein.

Cell-Dependent NOS Activity

Cell-dependent, stimulated NOS activity in cortical minces was also determined by a modification of the method of Bredt and Snyder (1989). The animals were killed by decapitation and their brains removed and placed into ice-cold, oxygenated (95% O_2, 5% CO_2) modified Krebs bicarbonate buffer containing 0.3 mM $CaCl_2$, 118 mM NaCl, 3.3 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 14.2 mM glucose, and 1.2 mM $MgSO_4$. The cortex was dissected and cross-chopped at 450 μm on a McIlwain tissue chopper. The minces were transferred to fresh ice-cold buffer and incubated on ice for 15 min. The buffer was changed and the minces were incubated at 37°C for 1 h with two additional buffer changes.

The minces were then transferred to a conical bottom tube on ice for gravity packing. Minivials were prepared that contained 30-μl aliquots of the appropriate drug. Freshly oxygenated buffer (270 μl) containing 30 nM L-[^{3}H]arginine (Amersham) was added. Fifty microliters of the gravity-packed slices were added in rapid succession to the minivials and incubated for 5 min. The reaction was stopped with 700 μl of an ice-cold solution of 4 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM unlabeled L-arginine dissolved in buffer. The minivials were then centrifuged at 1,000*g* and the supernatant decanted. The pellet was sonicated in 1 ml of 1 M trichloracetic acid (TCA). The vials were then spun at 12,000*g*, and an aliquot of the supernatant was collected and extracted three times with two volumes of ethyl ether to remove the TCA. The ether was allowed to evaporate overnight and a 0.5-ml aliquot of each sample was passed through 2 ml of a sodium equilibrated ion exchange resin (Dowex AG50W-X8) to separate the L-[^{3}H]citrulline formed from L-[^{3}H]arginine. L-[^{3}H]Citrulline was eluted in 4 ml of HEPES and combined with the sample effluent. L-[^{3}H]Arginine was eluted in 6 ml of HEPES at pH 12. This procedure eluted 98% of the total added radioactivity. Cell-dependent NOS activity is presented as [^{3}H]citrulline/[^{3}H]arginine + [^{3}H]citrulline × 100 minus a boiled tissue blank value divided by percent conversion of the same day untreated control.

RESULTS

All values in the text, tables, and figures are means ± standard error of the mean. Baseline mean arterial pressure (MAP) in the rats prepared for measurement of cell-dependent NOS activity in mince preparations was significantly higher than baseline MAP in the rats prepared for the crude soluble enzyme assay; however, there were no significant differences in MAP between the sham-injured and the injured groups. There were no differences among the groups in baseline (pretrauma) arterial oxygen, carbon dioxide, pH, or hemoglobin levels (Table 1). Levels of fluid-percussion TBI (2.2 atm, 22 ms) and MAP during transient arterial hypertension in response to TBI were the same in all groups (Table 1).

Total cortical soluble NOS activity was measured in control (n = 10), sham-injured (n = 5), or moderate TBI (n = 5) rats with either 0.45 or 1.5 μM added exogenous arginine (Fig. 1). There were no significant differences in soluble NOS activity between control, sham, or TBI groups at either concentration of added arginine. L-Nitroarginine methyl ester (L-NAME) blocked 97% of NOS activity when used to perfuse the brain during the surgery (25 mg/kg i.v.) or when added during the enzyme assay (30 μM). 3-Bromo-7-nitroindazole (30 μM) and *S*-methyl-thiocitrulline (10 μM), more specific inhibitors of the neuronal isoform of NOS, blocked 96% and 99% of NOS activity, respectively, when used during the enzyme assay (unpublished observations).

We have previously shown that 5-min incubation with 300 μM NMDA, 10 μM ionomycin or 50 mM KCl are able to significantly stimulate NOS activity in the cell-intact preparation each via different mechanisms (Ala-

TABLE 1. MEAN ARTERIAL BLOOD PRESSURE (MAP, mm Hg) AND ARTERIAL BLOOD GAS VALUES (mm Hg) AND HEMOGLOBIN (Hgb, g/dl) LEVELS IN RATS PREPARED FOR MEASUREMENT OF BASELINE OR STIMULATED NOS ACTIVITY AFTER MODERATE FLUID PERCUSSION TBI OR SHAM TBI

				Arterial blood			
Experiment	*Group*	*MAP baseline*	*MAP peak*	*pH*	*pO_2*	*pCO_2*	*Hgb*
Crude enzyme preparation	Sham	104.2 ± 1.4	—	7.38 ± 0.01	300 ± 16	40 ± 1	12.3 ± 0.2
	TBI	103.5 ± 1.7	140.7 ± 5.7	7.37 ± 0.01	303 ± 23	38 ± 1	12.8 ± 0.3
Mince preparation	Sham	121.3 ± 2.0	—	7.40 ± 0.01	311 ± 18	38 ± 1	12.3 ± 0.2
	TBI	126.5 ± 2.0	155.8 ± 7.6	7.40 ± 0.01	303 ± 13	37 ± 1	13.2 ± 0.2

Data are mean ± SEM.

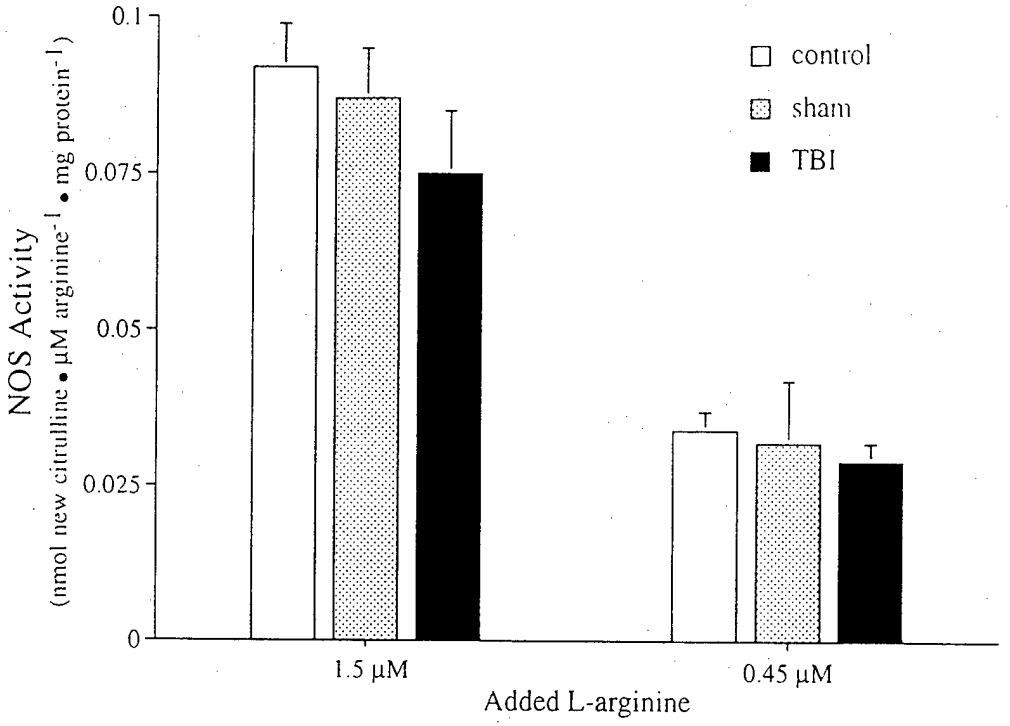

FIG. 1. Total baseline soluble nitric oxide synthase (NOS) activity determined from crude enzyme preparations from control (unoperated, $n = 10$) rats and rats subjected to sham injury ($n = 5$) or moderate ($n = 5$) fluid percussion traumatic brain injury (TBI).

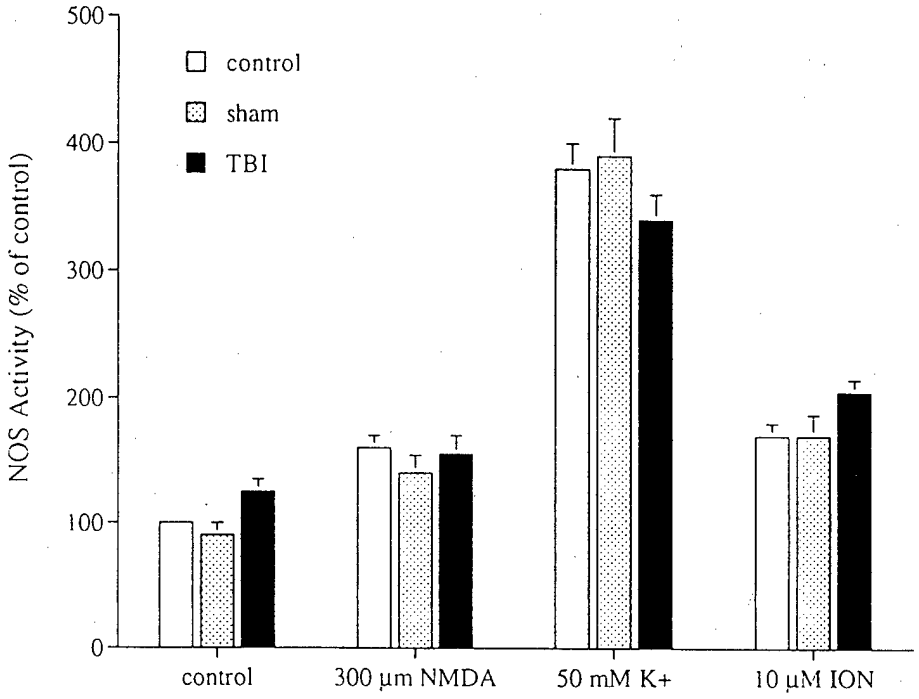

FIG. 2. Stimulated cell-dependent nitric oxide synthase activity (NOS) in mince preparations from control (unoperated, $n = 9$) rats and rats subjected to sham injury ($n = 5$) or moderate ($n = 5$) fluid-percussion traumatic brain injury (TBI) and then exposed to *N*-methyl-D-aspartate (NMDA), potassium chloride (K^+), or ionomycin (ION). Cit, citrulline; Arg, arginine.

garsamy et al., 1994). Since it is possible that cell-dependent NOS activity may be changed following TBI without affecting the total crude soluble activity, we compared baseline activity and NOS activity in response to the above stimuli. NOS activity in control ($n = 9$), sham injury ($n = 5$), and moderate TBI ($n = 5$) were not significantly different at either baseline or stimulated conditions (Fig. 2).

DISCUSSION

These studies demonstrated that moderate central fluid-percussion TBI did not significantly reduce basal soluble NOS activity or cell-dependent NOS activity in response to stimulation with the glutamate receptor agonist NMDA, to depolarization by KCl, or to a calcium ionophore (ionomycin).

Reduced CBF after TBI occurs in humans (Bouma et al., 1991; Kobayashi et al., 1991) as well as in experimental animals (Yamakami and McIntosh, 1989, 1991; Yuan et al., 1988; Armstead and Kurth, 1994). One possible mechanism by which this could occur is a reduction in the amount of NO, the major endothelium-derived relaxing factor (Ignarro et al., 1987), which is generated and released. Because the amount of NO that is released is directly proportional to the activity of NOS, it is possible that reduction of NOS activity would lead to a reduction in NO-mediated vasodilation. Reduction of NOS activity could be the result of enzyme destruction or an alteration in substrate or cofactor dependence after injury. Studies showing increased CBF after infusion of L-arginine, the NOS substrate, after TBI (DeWitt et al., 1997) suggest that changes in substrate availability may be the mechanism of reduced CBF. Our experiments failed to show any significant changes in total soluble NOS activity at either concentration of added substrate, suggesting that neither the amount of NOS nor its affinity for arginine was affected by moderate TBI.

This study was intended to test the hypothesis that posttraumatic hypoperfusion is due to reductions in NOS activity, rats were killed 30 min after injury or sham injury. Thirty minutes after moderate TBI is a time point when CBF is consistently reduced (Yuan et al., 1988; Yamakami et al., 1989; Muir et al., 1992; Dietrich et al., 1996). CBF returns to preinjury levels 2–4 h after TBI. In addition, hemorrhagic hypotension results in significant reductions in NOS activity 20 min after resuscitation (Kovach et al., 1994) and plasma nitrate/nitrite levels increase significantly 30 min after reperfusion after middle cerebral artery occlusion (Kimura et al., 1994). These studies indicate that NOS activity and NO metabolism change rapidly after cerebral injury, within the time period during which significant reductions in CBF occur after TBI.

Additionally, it is possible that NOS cofactor dependence or other changes related to calcium-mediated activation could lead to decreased NO synthesis. In this case, there would be a smaller response to drugs that stimulate NOS. We have previously shown that NMDA, K^+, and a calcium ionophore, ionomycin, each stimulate NOS activity by different calcium-dependent mechanisms (Alagarsamy et al., 1994). However, TBI had no effect on either basal or stimulated NOS activity in the cortex. These data suggest that TBI does not affect the ability of NOS to synthesize NO under a variety of conditions.

An alternate explanation is that the during preparation of the tissue, either during the surgery itself or during the preparation of tissue for the enzymatic assay, there was some artifact-induced reduction in baseline NOS activity. In this case, a further reduction in NOS activity due to TBI may not have been detected. It is possible that the isoflurane used during the surgical procedure may have significantly inhibited NOS activity (Terasako et al., 1994; Tobin et al., 1994). However, because the anesthetic was withdrawn 30 min prior to decapitation, the residual amount present in the *in vitro* preparations would have been minimal (Berg-Johnsen and Langmoen, 1987). Additionally, the levels of enzyme activity in both the crude enzyme preparation and the mince preparation were similar in the untreated animals and the sham surgery controls, suggesting that the isoflurane did not significantly inhibit NOS in our test preparations.

Although previous studies suggest that changes in substrate availability are a likely mechanism by which NOS activity might be altered (Morikawa et al., 1994; Fabricius et al., 1995), it is possible that sensitivity to the other cofactors necessary for NOS activation may be changed. Also, although the affinity of NOS for arginine may not be affected, it is possible that the availability of arginine is changed as a result of TBI. It is possible that TBI causes a reduction in the transport of arginine into the cells, thereby making arginine a limiting factor. Therefore, adding exogenous arginine may increase CBF after TBI (DeWitt et al., 1997) by helping to overcome reductions in transport efficiency. However, our experiments suggest that this may not be the case since, in the mince preparation, there were no differences in the total [^{3}H]arginine uptake in the various groups (data not shown).

Another possibility is that there are significant changes in NOS activity that are localized to discreet areas immediately surrounding the injury site. After central fluid-percussion injury, hypoperfusion occurs throughout the cerebral hemispheres (Yamakami and McIntosh, 1989, 1991; Yuan et al., 1988). However, other studies indicate that lateral fluid percussion TBI produces focal as well

as more widespread cerebral hypoperfusion in rats (Dietrich et al., 1996). Because we used the total cortex for our analyses, it is possible that reductions in NOS activity that occurred in small regions were diluted by surrounding areas in which NOS activity remained unchanged after TBI.

Brain temperature was not measured or controlled in this study. Although it is possible that brain temperature decreased after TBI, thereby protecting NOS from TBI-related damage, previous studies have reported on differences in brain temperature between fluid percussion TBI and sham injury in anesthetized rats (Jiang et al., 1991). However, TBI-induced brain temperature decreases don't prevent posttraumatic cerebral blood flow (CBF) decreases since previous studies have demonstrated that posttraumatic hypoperfusion occurs even if brain temperature is not controlled after TBI (Yuan et al., 1988; Yamakami et al., 1989; Muir et al., 1992). The important observation is that global NOS activity did not decrease 30 min after TBI, a time that is associated with significant reductions in CBF.

Although our data suggest that TBI does not affect total or stimulated NOS activity, it is still possible that there may be changes in NOS after TBI that were not detected. Although most NOS activity was blocked by the specific nNOS inhibitor, there was some residual activity. It is possible that this residual activity represented the pool of NOS that is sensitive to TBI. However, experiments attempting to identify changes in the residual pool were unsuccessful as the remaining activity was below the sensitivity limits of our assay.

These studies demonstrated that moderate central TBI does not affect basal or stimulated NOS activity. Although moderate TBI had no measurable effect on NOS activity, it is possible that a more severe TBI could have produced significant changes. Our present observations that NOS activity is unaffected by TBI, coupled with previous evidence that L-arginine improves CBF after TBI (DeWitt et al., 1997), suggest that TBI decreases NO levels by destroying NO directly, rather than by affecting NO production. This hypothesis is supported by evidence that TBI produces the superoxide anion radical (Wei et al., 1981; Fabian et al., 1995), which inactivates NO (Rubanyi and Vanhoutte, 1986) or rapidly converts it to the powerful oxidant, peroxynitrite (Beckman, 1991, 1994). Further studies involving direct measurements of NO levels after TBI are required to determine the effects of TBI on NO within the central nervous system and the cerebral vasculature.

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REFERENCES

ALAGARSAMY, S., LONART, G., and JOHNSON, K.M. (1994). Regulation of nitric oxide synthase activity in cortical slices by excitatory amino acids and calcium. J. Neurosci. Res. **38,** 648–653.

ARMSTEAD, W.M., and KURTH, C.D. (1994). Different cerebral hemodynamic responses following fluid percussion brain injury in the newborn and juvenile pig. J. Neurotrauma **11,** 487–497.

BECKMAN, J.S. (1991). The double-edged role of nitric oxide in brain function and superoxide-mediated injury. J. Dev. Physiol. **15,** 53–59.

BECKMAN, J.S., CHEN, J., CROW, J.P., and YE, Y.Z. (1994). Reactions of nitric oxide, superoxide and peroxynitrite with superoxide dismutase in neurodegeneration. Prog. Brain Res. **103,** 371–380.

BECKMAN, J.S., CONGER, K.A., CHEN, J., TAN, M.J., and HALSEY, J.H. (1991). Nitric oxide helps regulate cerebral blood flow. J. Cereb. Blood Flow Metab. **11,** S629.

BERG-JOHNSEN, J., and LANGMOEN, I.A. (1987). Isoflurane hyperpolarizes neurones in rat and human cerebral cortex. Acta Physiol. Scand. **130,** 679–685.

BOUMA, G.J., MUIZELAAR, J.P., CHOI, S.C., NEWLON, P.G., and YOUNG, H.F. (1991). Cerebral circulation and metabolism after severe traumatic brain injury: the elusive role of ischemia. J. Neurosurg. **75,** 685–693.

BOUMA, G.J., MUIZELAAR, J.P., STRINGER, W.A., CHOI, S.C., FATOUROS, P., and YOUNG, H.F. (1992). Ultra-early evaluation of regional cerebral blood flow in severely head-injured patients using Xenon-enhanced computerized tomography. J. Neurosurg. **77,** 360–368.

BREDT, D.S., and SNYDER, S.H. (1989). Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc. Natl. Acad. Sci. USA **86,** 9030–9033.

CROW, J.P., and BECKMAN, J.S. (1995). Reactions between nitric oxide, superoxide and peroxynitrite: footprints of peroxynitrites *in vivo*. Adv. Pharmacol. **34,** 17–43.

DEWITT, D.S., KONG, D.L., LYETH, B.G., et al. (1988). Experimental traumatic brain injury elevates brain prostaglandin E_2 and thromboxane B_2 levels in rats. J. Neurotrauma **5,** 303–313.

DEWITT, D.S., PROUGH, D.S., COLONNA, D.M., DEAL, D.D., and VINES, S.M. (1992). Effects of nitric oxide synthase inhibitors on cerebral blood flow and autoregulation in rats. Anesthesiology **77,** suppl 3A, A689(abstract).

DEWITT, D.S., SMITH, T.G., DEYO, D.J., MILLER, K.R., UCHIDA, T., and PROUGH, D.S. (1997). L-Arginine and superoxide dismutase prevent or reverse cerebral hypoperfusion after fluid-percussion traumatic brain injury. J. Neurotrauma **14,** 219–229.

DIETRICH, W.D., ALONSO, O., BUSTO, R., PRADO, P., DEWANJEE, S., DEWANJEE, M., and GINSBERG, M.D. (1996). Widespread hemodynamic depression and focal platelet accumulation after fluid percussion brain injury: a double-label autoradiographic study in rats. J. Cereb. Blood Flow Metab. **16,** 481–489.

DIXON, C.E., LYETH, B.G., POVLISHOCK, J.T., et al. (1987). A fluid percussion model of experimental brain injury in the rat. J. Neurosurg. **67,** 110–119.

FABIAN, R.H., DEWITT, D.S., and KENT, T.A. (1995). In vivo detection of superoxide anion production by the brain using a cytochrome c electrode. J. Cereb. Blood Flow Metab. **15,** 242–247.

FABRICIUS, M., AKGOREN, N., and LAURITZEN, M. (1995). Arginine-nitric oxide pathway and cerebrovascular regulation in cortical spreading depression. Am. J. Physiol. **269,** H23–H29.

FURCHGOTT, R.F., and ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature **288,** 373–376.

GRAHAM, D.I., ADAMS, J.H., and DOYLE, D. (1978). Ischaemic brain damage in fatal non-missile head injuries. J. Neurol. Sci. **39,** 213–234.

IGNARRO, L.J., BUGA, G.M., WOOD, K.S., BYRNS, R.E., and CHAUDHURI, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. USA **84,** 9265–9269.

JIANG, J.Y., LYETH, B.G., CLIFTON, G.L., JENKINS, L.W., HAMM, R.J., and HAYES, R.L. (1991). Relationship between body and brain temperature in traumatically brain-injured rodents. J. Neurosurg. **74,** 492–496.

KOBAYASHI, H., HAYASHI, M., KAWANO, H., HANDA, Y., KABUTO, M. and IDE, H. (1991). Effect of chemical sympathectomy on cerebral blood flow in rats. J. Neurosurg. **75,** 906–910.

KOVACH, A.G.B., LOHINAI, Z., MARCZIS, J., BALLA, I., DAWSON, T.M., and SNYDER, S.H. (1994). The effect of hemorrhagic hypotension and retransfusion and 7-nitro-indazole on rCBF, NOS catalytic activity, and cortical NO content in the cat. Ann. N.Y. Acad Sci. **738,** 348–368.

KUMURA, E., KOSAKA, H., SHIGA, T., YOSHIMINE, T., and HAYAKAWA, T. (1994). Elevation of plasma nitroc oxide end products during focal cerebral ischemia and reperfusion in the rat. J. Cereb. Blood Flow Metab. **14,** 487–491.

MORIKAWA, E., MOSKOWITZ, M.A., HUANG, Z., YOSHIDA, T., IRIKURA, K., and DALKARA, T. (1994). L-Arginine infusion promotes nitric oxide-dependent vasodilation, increases regional cerebral blood flow, and reduces infarct volume in rats. Stroke **25,** 429–435.

MUIR, J.K., BOERSCHEL, M., and ELLIS, E.F. (1992). Continuous monitoring of posttraumatic cerebral blood flow using laser-Doppler flowmetry. J. Neurotrauma **9,** 355–362.

PELLIGRINO, D.A., KOENIG, H.M., and ALBRECHT, R.F. (1993). Nitric oxide synthesis and regional cerebral blood flow responses to hypercapnia and hypoxia in the rat. J. Cereb. Blood Flow Metab. **13,** 80–87.

RUBANYI, G.M., and VANHOUTTE, P.M. (1986). Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. Am. J. Physiol. **250,** H822–H827.

TANAKA, K., GOTOH, F., GOMI, S., et al. (1991). Inhibition of nitric oxide synthesis induces a significant reduction in local cerebral blood flow in the rat. Neurosci. Lett. **127,** 129–132.

TERASAKO, K., NAKAMURA, K., MIYAWAKI, I., TODA, H., KAKUYAMA, M., and MORI, K. (1994). Inhibitory effects of anesthetics on cyclic guanosine monophosphate (cGMP) accumulation in rat cerebellar slices. Anesth. Analg. **79,** 921–926.

TOBIN, J., MARTIN, L., BRESLOW, M., and TRAYSTMAN, R. (1994). Selective anesthetic inhibition of brain nitric oxide synthase. Anesthesiology **81,** 1264–1269.

WEI, E.P., KONTOS, H.A., DIETRICH, W.D., POVLISHOCK, J.T., and ELLIS, E.F. (1981). Inhibition by free radical scavengers and by cyclooxygenase inhibitors of pial arteriolar abnormalities from concussive brain injury in cats. Circ. Res. **48,** 95–103.

YAMAKAMI, I., and McINTOSH, T.K. (1989). Effects of traumatic brain injury on regional cerebral blood flow in rats as measured with radiolabeled microspheres. J. Cereb. Blood Flow Metab. **9,** 117–124.

YAMAKAMI, I., and McINTOSH, T.K. (1991). Alterations in regional cerebral blood flow following brain injury in the rat. J. Cereb. Blood Flow Metab. **11,** 655–660.

YUAN, X.Q., PROUGH, D.S., SMITH, T.L., and DEWITT, D.S. (1988). The effects of traumatic brain injury on regional cerebral blood flow in rats. J. Neurotrauma **5,** 289–301.

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